The characterisation of human regulatory T cell subsets in ageing and atopy

Nicola Jane Booth

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Department of Immunology and Molecular Pathology
Division of Infection and Immunity
University College London
46 Cleveland Street
London W1T 4JF
United Kingdom

I, Nicola Jane Booth, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this is indicated in the thesis.

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Dedication

This work is dedicated to my parents, Steve and Carolyn Booth.

Abstract

The immune system must be controlled to prevent damage caused by inappropriate responses and extended inflammation. Regulatory T cells (Tregs), known to be generated by the thymus, must be maintained in the face of an ever-increasing human lifespan and associated thymic atrophy in order to protect the host, but whether they are maintained by expansion of pre-existing Tregs or conversion of conventional T cells is not yet known.

There are known to be two subsets of FOXP3+ regulatory T cells: naive and memory cells, expressing CD45RA and CD45RO respectively. In this work the characteristics of CD45RA+ and CD45RO+ regulatory T cells were investigated in healthy adults. We found proliferative and phenotypic differences between the two subsets, and evidence that CD45RA+ Tregs can replenish the memory Treg pool on activation. It is, however, becoming more accepted that CD45RO+ Tregs are also likely to be composed of many cells that were converted externally to the thymus from conventional T cells, and our work suggests a mechanism for this conversion: anergy induction. We also found that the two Treg subsets are able to migrate to disparate tissues. Investigation of cutaneous immune responses *in vivo* revealed the presence of a significant proportion of Tregs, their numbers rising and falling in concordance with the number of conventional T cells.

Finally, these investigations of Treg subsets were extended to investigate atopic dermatitis (AD), a hypersensitivity condition in which Tregs are implicated. We found significantly fewer CD45RA+ Tregs among AD patients, with unexpectedly low rates of turnover of these cells in AD skin, despite the presence of high proportions of CD4+FOXP3+ cells.

Overall, the findings from this study imply disparate roles for CD45RA+ and CD45RO+ Tregs, and provide further evidence supporting a role for dysregulated regulatory T cell function in the pathogenesis of atopic dermatitis.

Abbreviations

APC	Antigen presenting cell
APC	Allophycocyanin
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CNS	Central nervous system
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
Cpm	Counts per minute
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EASI	Eczema Area and Severity Index
E-selectin	Endothelial cell selectin
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box protein 3
FSC	Forward scatter
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

LAD	Leukocyte adhesion deficiency
LC	Langerhans cell
LFA	Leukocyte function-associated antigen
LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK cell	Natural killer cell
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECy7	Phycoerythrin-cyanin 7
PerCP	Peridinin chlorophyll protein
PPD	Tuberculin purified protein derivative
P-selectin	Platelet selectin
PRRs	Pattern recognition receptors
RBC	Red blood cell
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SSC	Side scatter
Tregs	Regulatory T cells
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TREC	T cell receptor excision circle

Publications, abstracts and awards

Publications

NJ Booth, AJ McQuaid, TO Sobande, S Kissane, E Agius, S Jackson, M Salmon, F Falciani, K Yong, MH Rustin, AN Akbar, M Vukmanovic-Stejic. Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO. **Journal of Immunology,** April 2010, **184**(8):4317-26

M Vukmanovic-Stejic, E Agius, **N Booth**, PJ Dunne, KE Lacy, JR Reed, TO Sobande, S Kissane, M Salmon, MH Rustin, AN Akbar. The kinetics of CD4+FOXP3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo. **Journal of Clinical Investigation.** November 2008, **118**(11):3639-50

Presentations

Natural Treg hetereogeneity in humans with and without atopic dermatitis. **N Booth,** AJ McQuaid, T Sobande, E Agius, K Yong, M Rustin, AN Akbar, M Vukmanovic-Stejic. Poster presented at *Regulatory T Cells* Keystone Symposium, Keystone, March 2009

Distinct proliferation rates and localisation of naïve and memory Tregs in healthy humans *in vivo*. **N Booth,** A McQuaid, T Sobande, E Agius, K Yong, M Rustin, AN Akbar, M Vukmanovic-Stejic. Oral presentation given at EAACI- Ga^{2}len Immunology Winter School, Davos, February 2009.

Distinct proliferation rates and localisation of naïve and memory Tregs in healthy humans *in vivo.* **N Booth,** AJ McQuaid, T Sobande, E Agius, K Yong, M Rustin, AN Akbar, M Vukmanovic-Stejic. Poster presented at the British Society for Immunology Congress, Glasgow, November 2008.

Awards

1. **European Academy of Allergy and Clinical Immunology** travel award to attend and present at the EAACI Immunology Winter School, Davos, Switzerland, February 2009; value €250.00
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3. **Keystone Symposia** scholarship to attend and present a poster at the Keystone Symposium on Regulatory T cells, Colorado, March 2009; value $1000.00

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2. Vukmanovic-Stejic, M. et al. *The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo.* J. Clin. Invest. 2008. **118:**3639-3650

3. Endothelial transmigration time-lapse videos (attached CD and at www.mediafire.com/migrationvideos)

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Chapter 1. Introduction

1.1 The immune system

The immune system relies on a complex interplay of cells and molecules in order to protect the host from infection by pathogens. Although barriers, such as skin and lysozyme-containing saliva, do exist, they are occasionally breached by infectious organisms such as viruses and bacteria. There are many different types of pathogen, all with different strategies for persisting within the host; as a result, the immune system employs a number of different cell types with different specialised functions.

All immune cells, or leukocytes [1], are ultimately generated in the bone marrow from haematopoietic stem cells [2]. However, they subsequently develop via differing routes of maturation and play discrete roles within the immune system. Innate immune cells, such as neutrophils, macrophages, eosinophils and NK cells, are unable to recognise a specific antigen from a particular pathogen, but are able to recognise that an organism is likely to be dangerous. They respond quickly to infection: neutrophils arrive at the site of pathogen invasion within the first few hours [3]. T cells and B cells form part of the adaptive arm of the immune system: they are highly varied and each individual B or T cell is specific for a slightly different antigen [4, 5]. They are initially slow to respond to primary infection, as the rare cells specific for the required antigens must proliferate before they can reach sufficient numbers to be effective. However, if the pathogen invades the host a second time, a much faster secondary immune response is generated by the adaptive immune system because of the memory cells generated during prior exposure [6].

Neutrophils and macrophages are phagocytic cells: they engulf, and subsequently digest, pathogens [7] which they, like many innate cells, recognise by the presence of pathogen-associated molecular patterns (PAMPS). These include bacterial products such as lipopolysaccharide (LPS) from the microbe cell wall, viral products such as double-stranded RNA and fungus-specific molecules, like zymosan [8]. PAMPs are detected by pattern-recognition receptors (PRRs) of which the most numerous are toll-like receptors (TLRs) [8, 9]. Macrophages are also able to control the conduct of the subsequent immune response, by releasing the appropriate cytokines to favour either a cell-mediated or an antibody-mediated response. Other innate immune cells include eosinophils, which are, like neutrophils, phagocytic [10] but also contain granules which

are secreted on activation to release inflammatory mediators [11]. They are thought to be useful in countering parasites such as helminths, as well as wound-healing [12] and play a prominent role in many allergic reactions [13-15]. Eosinophils are attracted by chemicals released from mast cells, another form of innate immune cell which contains granules that, on secretion, release factors including histamine; this increases the permeability of venules, allowing cells to exit the bloodstream more easily [16].

Natural killer cells are lymphocytes [17]. They do not, however, recognise specific antigen but rather detect virally-infected cells because of an absence of certain surface markers [18]. They then destroy the cells and the nascent virus within, via release of perforin and granzyme, which perforate the cell membrane and initiate apoptosis of the infected cell [19, 20].

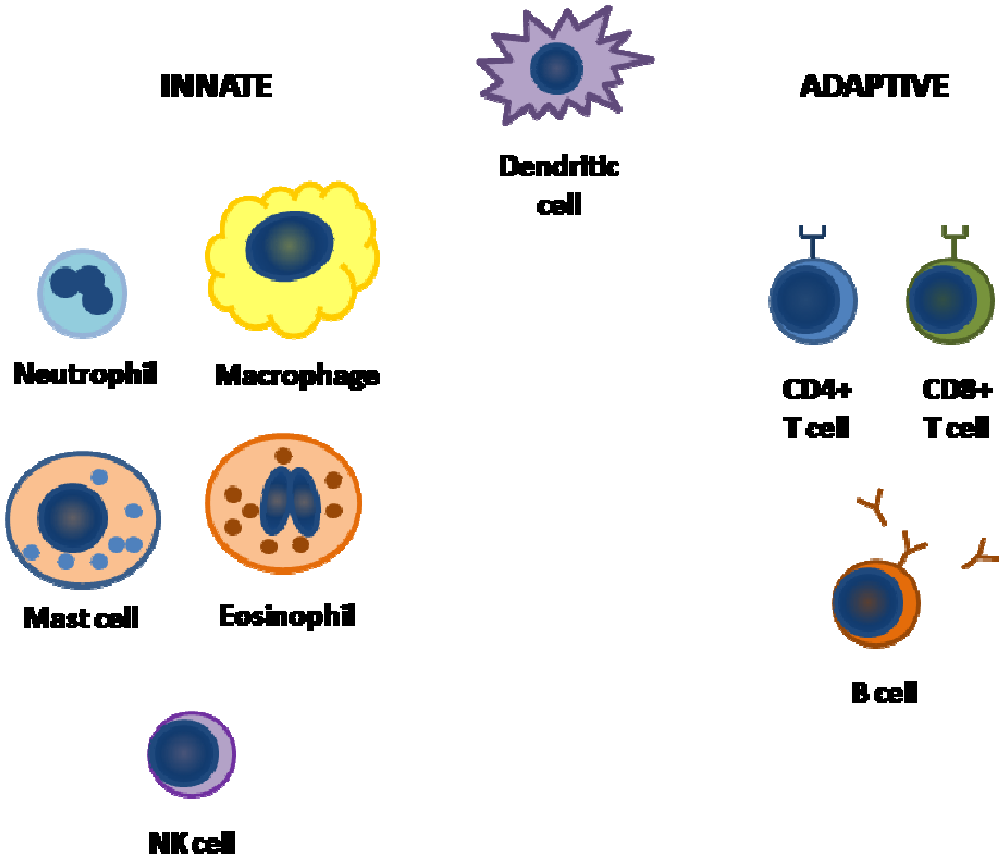


Figure 1.1. The immune system.

The immune system can be divided into innate (left) and adaptive (right) cells, with dendritic cells (centre) interacting with both. Innate cells recognise pathogens by their shared expression of Pathogen-Associated Molecular Patterns (PAMPs) which are detected by Toll-like receptors. Adaptive immune cells possess highly specific receptors: T cells possess a T cell receptor (TCR) and B cells have a BCR, which can be secreted as antibody.

The gap between the innate and adaptive immune systems is bridged by dendritic cells (DCs) [21, 22]: although not capable of forming memory cells, they are able to signal to T and B cells to alert them to infection. These cells are found in their immature state in the tissues [23] and possess long cell extensions [22] with which they sweep the environment; if they detect a pathogen or foreign cell, they become activated, migrating to the lymph nodes and presenting antigens from the pathogens to T or B cells [24, 25]; hence, they are termed antigen-presenting cells (APCs) [25]. Although activated DCs are able to initiate an immune response, immature DCs have more recently been found to exert tolerogenic effects on the immune system, inducing IL-10 production and regulatory (suppressive) function in T cells they interact with [26, 27].

B cells and T cells are lymphocytes, like natural killer cells, and circulate in the bloodstream and the lymphoid system to access lymph nodes**.** These are compartments allowing the interaction of (normally tissue-resident) antigen presenting cells and lymphocytes, which enter the lymph node from the bloodstream via high endothelial venules [28]. Once the lymphocytes are activated, they migrate to the original source of the antigen (the site of infection) and destroy the pathogen.

B cells develop in the bone marrow and are able to produce antibody, or immunoglobulin (Ig): the B cell receptor (BCR) is surface-bound immunoglobulin [29]. There is an extraordinarily large variety of potential BCRs, all with slightly different specificities [30], resulting from the capacity of B cells to rearrange the DNA encoding their surface antigen receptors; T cells also have this ability, resulting in a broad spectrum of T cell receptor (TCR) specificities as well [31, 32]. BCRs recognise a protein in its natural conformation. Once activated, by recognising its specific (cognate) antigen, the B cell is stimulated to divide many times and undergoes a process termed somatic hypermutation, whereby the BCR is mutated slightly each time: those cells possessing new BCRs with the highest specificity for the antigen divide again and so on, until the affinity of the receptor for the antigen is much stronger [29]. The B cells generated by this process are conventionally accepted to continue down one of two different routes: they can generate plasma cells, which generate and secrete large quantities of antibody, or memory B cells, which can participate in future reactions [33]. Secreted antibody has the same specificity as the plasma cell's BCR and binds to its cognate antigen at its variable (F_{ab}) region [34]. The other end of the antibody is termed the constant (F_c) region [34, 35] and can bind to receptors on other immune cells such as macrophages and neutrophils; when many antibodies are bound to one antigen, their F_c regions stimulate engulfment and destruction of the pathogen [36].

T cells exist in two major subgroups: CD4-expressing (CD4+) ‘helper’ T cells and CD8+ ‘killer’ (cytotoxic) T cells, which recognise antigenic peptides bound to different MHC [37]. CD8+ T cells recognise peptides in the context of MHC class I (HLA-A, B or C). They secrete IFN-γ, express perforin and granzyme and act to destroy infected or cancerous cells. CD4+ T cells are MHC class II-restricted, recognising peptides bound to HLA-DR, DP or DQ. They are not usually thought to kill directly but instead play a more controlling role in the immune response, providing help to activate both B cells and CD8+ T cells to perform their roles. They can also produce a range of cytokines, influencing the course of the subsequent immune response [38, 39].

Activated T cells, like B cells, can become long-lived memory cells or more short-lived effector cells [40]. The ability of lymphocytes to generate long-lived cells with a highly-specific receptor forms the basis of the phenomenon known as immunological memory. As noted above, naïve cells are difficult to activate initially; there are also very few naïve T cells able to recognise antigens from the same pathogen, and the same is true of B cells. This means that the (primary) adaptive immune response to a pathogen on initial encounter is relatively slow. However, circulating memory cells of a particular specificity are both higher in number and easier to activate than naïve cells, and also patrol the peripheral lymph nodes and tissues [40], enabling a secondary immune response to be much more rapid, often preventing the infection from taking hold altogether. The individual is hence said to be immune.

Immunological memory is exploited in the field of medicine via vaccination, whereby attenuated or killed pathogens, or pathogen components, are injected into the individual, enabling the immune system to recognise and eradicate the pathogen if it should be encountered again [41].

1.1.1 CD4+ T cells

Whereas CD8+ T cells release perforin and granzyme to destroy the infected cell, the primary function of CD4+ T cells is not killing (although they have been shown to be capable of this [42-44]). They specialise in the provision of ’help’ to both B cells and cytotoxic T cells, to enable these cell types to function more efficiently and generate memory populations [44-46]. CD4+ T cells are also able to skew the immune response depending on the combination of cytokines they release on activation: for example, Th1 (T-helper 1) cells favour a cell-mediated, cytotoxic T-cell response and Th2 cells favour

the activation of eosinophils and a humoral response [38, 39]. CD4+ T cells are the main focus of work contributing to this thesis.

Generation and activation

T cell generation is initiated in the bone marrow, but the developing cells migrate to the thymus to complete their maturation; while still in the thymus, they are termed thymocytes. As is the case with B cells, thymocytes somatically rearrange the DNA encoding their TCR in order to generate a large range of potential TCR specificities [32]. This rearrangement generates small circles of excised DNA, called T cell receptor excision circles (TRECs). These are not replicated as the cell divides, so cell populations with a higher TREC content can be assumed to have divided fewer times since their generation [47]. High TREC content therefore suggests a naïve T cell population.

Once the novel TCR has been generated on each cell, its specificity is tested. T cell receptors recognise short stretches of peptide from the antigen only in the context of the MHC (major histocompatibility complex) [48, 49]. MHC class I presents peptides to CD8+ T cells, whereas CD4+ T cells are generally MHC class II-restricted, although exceptions to this have been reported [50].In the thymus, maturing thymocytes encounter MHC presenting self-antigen on thymic epithelial cells [51]. If the thymocyte does not recognise the MHC+peptide complex at all, it will die from neglect, whereas those thymocytes which respond to the presence of MHC receive survival signals; this is called positive selection. If it recognises it too strongly (and hence has the potential to be autoreactive), it is generally deleted: this process is termed negative selection [52-54]. Cells which react with low affinity for self peptide and MHC continue to mature and are released from the thymus as naïve T cells [55]. There are thought to be exceptions to this: sometimes, it is thought that strongly-reacting thymocytes may become anergic (unresponsive) or undergo receptor editing, or possibly become suppressive regulatory T cells (Tregs) [55, 56].

MHC molecules present peptides from processed proteins: MHC class I peptides are normally from proteins synthesised within the cell, whereas MHC class II peptides are generated via degradation of proteins within endosomal compartments [57]. Under normal conditions these are self-peptides, eliciting no immune response from the previously-tolerised circulating T cells. However, virally-infected cells display viral peptides on MHC class I. A very small number of mature CD8+ T cells will be able to recognise the combination of peptide and MHC molecule and will hence become

activated and proliferate. Likewise, external antigens can be ingested and processed by professional antigen-presenting cells, and their peptides presented on MHC class II. This will activate the small proportion of CD4+ T cells whose TCRs recognise that particular peptide-MHC combination.

TCR stimulation alone is not sufficient to completely activate mature T cells [58]. They require additional costimulatory signals, for example from CD28, which binds the dendritic cell-expressed molecules B7-1 (CD80) and B7-2 (CD86) [59]. These costimulatory molecules, along with signalling molecules and the CD4 or CD8 coreceptor, cluster around the TCR as the T cell and APC form a stable interaction called the immunological synapse (IS) [60, 61].

CTLA-4 is also present in the IS structure. This is an inhibitory molecule, which also binds B7-1 and -2 but with a much higher affinity than CD28 [59]. Expression of CTLA-4 is tightly regulated: it is not present on resting CD4+ conventional T cells and must be synthesised *de novo* after activation [59]. Once it has been upregulated at the surface, it is rapidly endocytosed again: this tightly-regulated cycling provides a check on CTLA-4 surface expression levels [62]. Although CTLA-4 is known to inhibit T cell activation, a supposition supported by the fatal lymphoproliferative syndrome seen in CTLA-4 knockout mice [63], the precise mechanism by which it inhibits activation is still a matter of debate [59].

Naïve T cells require extremely strong costimulatory signals in order to be activated for the first time, which can only be provided by a professional antigen-presenting cell (APC) – conventional DCs are the only cells so far discovered to have this ability [21]. These controls help to further reduce the possibility that self-reactive cells could become activated and attack the host.

Differentiation

T cells leaving the thymus directly after maturation are referred to as ‘naïve’ and express the long splice variant of CD45, CD45RA [64-67]. They also express high levels of the costimulatory receptors CD27 and CD28 [64-66, 68] as well as homing markers that direct them to the lymph nodes: CD62L and CCR7 [64]. It has been proposed that naïve cells can divide around two to three times before losing expression of CD45RA [69, 70]. ‘Truly naïve’ recent thymic emigrants (RTEs) have been suggested to be identifiable by their coexpression of CD45RA and CD31, an adhesion molecule also known as platelet endothelial cell adhesion molecule-1 (PECAM-1) [69,

71]. CD45RA+CD31+ T cells have been shown to have longer telomeres and higher quantities of TRECs (T cell receptor excision circles, formed when the maturing thymocyte rearranges its T cell receptor (TCR) and diluted with proliferation of the pool) than the CD45RA+CD31- pool [69], suggesting that these cells have divided fewer times.

After encountering cognate antigen, these cells begin to proliferate and undergo a process of differentiation, where these surface proteins are gradually replaced with others. As they differentiate they become known as central memory and then effector memory cells (see Table 1) [40]. CD45RA is downregulated and the shortest splice variant of CD45, CD45RO, begins to be expressed instead [64, 65, 67], and CD27 and CD28 are sequentially lost as the cell becomes more differentiated (CD8+ T cells, however, lose CD28 first) [64, 66-68].

As their surface phenotype alters, so does their function. Naïve CD4+ T cells produce large amounts of interleukin 2 (IL-2), a cytokine that encourages T cell survival and growth [72]. However, on stimulation they are thought to follow distinct pathways of differentiation to become one of four helper T cell subsets, producing different cytokines, depending on cues they receive from the stimulating antigen presenting cell [73]. Th1 cells produce large quantities of the pro-inflammatory cytokine interferon-gamma (IFN-γ), useful in viral infections and for clearing intracellular bacterial infections [38]. Th2 cells, in contrast, produce IL-4 and IL-13, which favour an antibody-mediated response [38, 39]. CD4+ T cells can also differentiate into Th17 cells, which produce the pro-inflammatory cytokine IL-17, thought to be beneficial in combating viral and fungal infections but also implicated in many autoimmune conditions [74-76]. Finally, CD4+ T cells have also been suggested to differentiate into suppressive regulatory T cells under certain conditions [77, 78]. CD8+ T cells also change function as they differentiate: they lose their capacity to generate IL-2 [79] and have been found to become increasingly cytotoxic as they become more differentiated [68, 80] as well as releasing larger quantities of IFN-γ [68].

One other gradual change taking place as T cells differentiate and divide is their loss of telomeric repeats at the end of chromosomes. Telomeres are long sections of DNA at each end of the chromosome containing only hexameric (GGGTTA) repeats and are gradually eroded with repeated divisions of the cell; they exist to prevent the loss of coding genetic material from the DNA strand, as well as to maintain stability of the chromosome [81]. The loss of genetic material from chromosomes on their replication

results from the linear nature of eukaryotic DNA: DNA polymerase, the enzyme that builds the new strands of DNA using the existing strands as a template, cannot quite replicate the nucleic acid to the end of the strand [82]. It can only copy DNA in a 5' to 3' direction (see Figure 1.2); paired DNA strands run in opposite directions, so one must be replicated in short sections, called Okazaki fragments, as the DNA opens [83]. DNA polymerase uses an RNA primer to initiate each new DNA strand or fragment, which is then removed from the template strand, leaving a gap. This gap can be filled by an exonuclease and DNA ligase, but the gap at the very end of the strand cannot be filled, so a 3' overhang is left. As a result, some genetic material is lost from the ends of the chromosomes with each cell division [82]; this is called the 'end-replication problem'. Once the telomeres have been eroded to a certain level (the Hayflick limit [84-86]), the cell is prevented from dividing further and apoptoses. Stem cells contain the telomere-lengthening enzyme telomerase [87, 88], which can also be upregulated in activated T cells [89]. This can repair the telomeres via an RNA template [90] and hence allow the cell to divide more times or even indefinitely [91]. However, telomerase is turned off when T cells become highly differentiated [92, 93], which limits their proliferative capacity.

Table 1.1. Changing phenotype with increasing differentiation of T cells

Phenotype	Naïve	Central memory	Effector memory	CD45RA Memory	References
CD45RA	+++	-	-	+++	[64-67]
CD45RB	+++	+++	+	+	[64, 65]
CD45RO	-	+++	+++	-	[64, 65, 67]
CD28	+++	++	+ (CD4) - (CD8)	+/-	[64, 66-68]
CD27	+++	++	- (CD4) + (CD8)	+/-	[64-66]
CCR7	+++	++	-	-	[64, 66]
CD62L	+++	+++	+	+	[64, 94]
LFA-1	-	+++	+++	+++	[95, 96]
CD95	-	+++	+++	+++	[97]
CTLA-4	+++	++	++	+	[98, 99]
PD-1	+	+++	+++	++	[100, 101]
KLRG-1	+	++	++	+++	[102, 103]
BCL-2	+++	++	+	++	[94, 95, 97, 104]
Telomere length	+++	++	+	++	[95, 105]

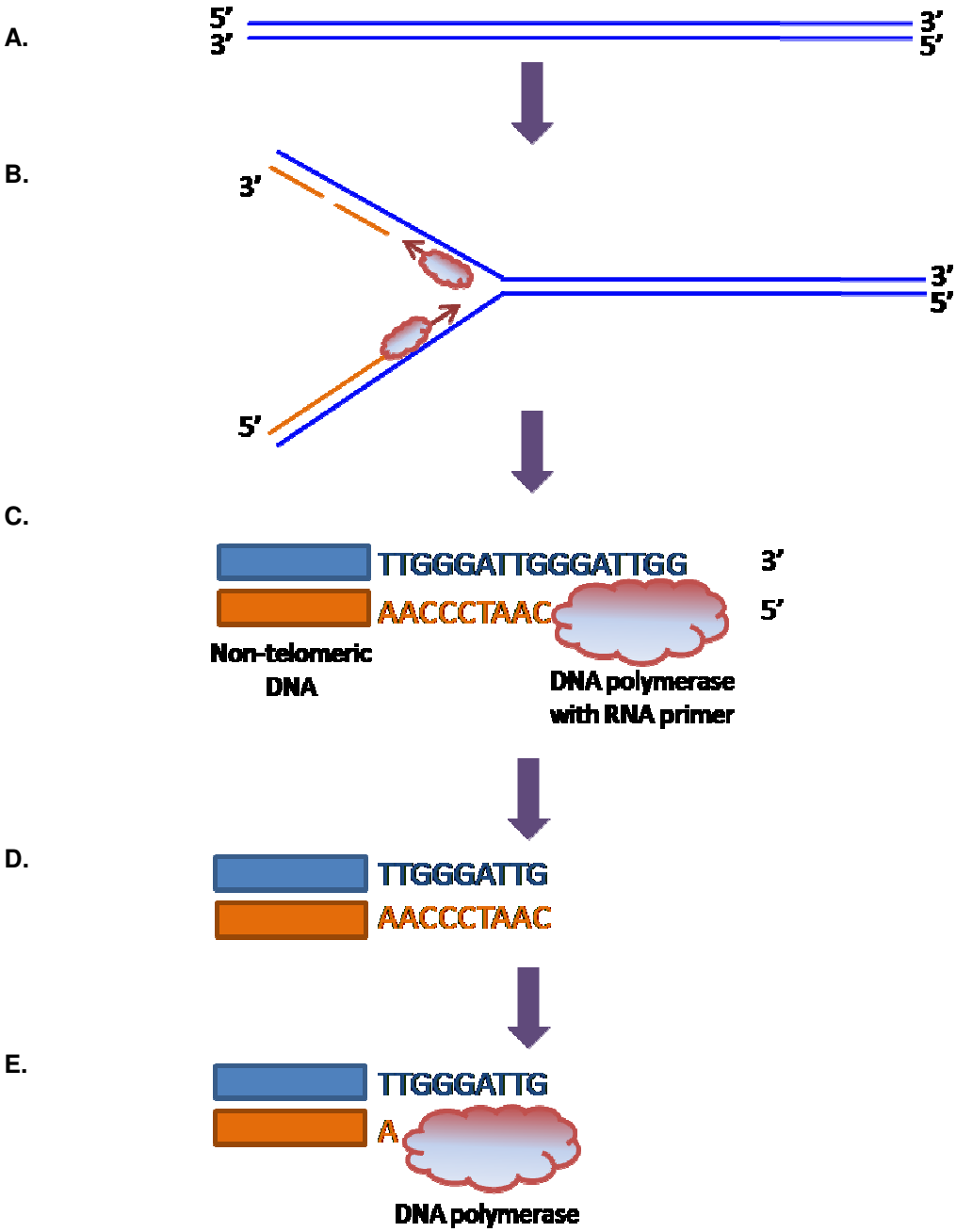


Figure 1.2. The end-replication problem, telomeres and the Hayflick limit.

The linear character of eukaryotic DNA means that DNA polymerase cannot replicate each chromosome to its very end. **A.** Paired DNA strands run in opposite directions, 5'-3' and 3'-5'. **B.** DNA polymerase can only replicate DNA in a 5'-3' direction. As a result one strand is replicated continuously (the leading strand) while the other, lagging, strand is replicated in short stretches, called Okazaki fragments, which are subsequently ligated together. **C.** DNA polymerase is unable to replicate to the very end of the strand, resulting in a gradual shortening of the genetic material (**D** and **E**). Repeating telomeric repeats protect the coding DNA but after these have been eroded to a particular degree, the cell is unable to proliferate further: this is known as the Hayflick limit.

Migration

Naïve T cells are known to circulate in the blood and lymphatic system, homing preferentially to the lymph nodes. However, once activated, T cells upregulate specific receptors that allow them to home to inflamed tissue, exiting the bloodstream at the appropriate site and following a gradient of chemokines to the precise area of inflammation [106].

Transendothelial migration

In order to pass from the bloodstream to the tissue, leukocytes must cross the barrier posed by the wall of the blood vessel, composed of a continuous layer of endothelial cells. Migration through endothelium is a multistep process, involving rolling of the cell along the endothelial surface, firm adhesion to the blood vessel wall and migration of the cell through the endothelial layer, a process termed diapedesis [107] (see Figure 1.3). This process has been extensively studied with respect to both lymphocytes and granulocytes but will be discussed here specifically in the context of T cell migration.

The first stage of T cell migration through endothelium involves the cell rolling along the vessel wall, forming transient attachments which are formed rapidly and break as the cell moves away, but serve to slow the cell down against the shear force of the blood flow [106, 108]. These attachments are mediated by the interaction of selectins with their ligands; the binding sites for selectins are carbohydrate moieties, including sialyl-Lewis^X [108, 109]. There are three selectins involved in leukocyte rolling: L-selectin (CD62L), P-selectin (CD62P) and E-selectin (CD62E) [109-112]. L-selectin is expressed by the leukocytes themselves, whereas E-selectin is expressed on endothelial cells only when activated by, for example, IL-1 or TNF [113]. P-selectin is stored in secretory granules located in the cytoplasm of endothelial cells and platelets and is cycled to the cell membrane on activation of the cells [114]. Its synthesis is also increased by some inflammatory mediators such as LPS (lipopolysaccharide) [115].

L-selectin is able to bind a number of different ligands, including CD34 [116] and the mucosal vascular addressin MadCAM1 [117]. Ligands for L-selectin, including CD34, are constitutively expressed by endothelial cells in high endothelial venules, which run through lymph nodes, Naïve cells have high expression of L-selectin (see Table 1.1); this allows them to home to secondary lymphoid organs for priming [118]. The ligands are also, however, upregulated on endothelial cells at sites of inflammation [119]. P-selectin and E-selectin bind ligands expressed on the surface of the T cell; in the case

of E-selectin, this is known to include cutaneous lymphocyte antigen (CLA) [120, 121] and CD66 [122], an inhibitory receptor upregulated after T cell activation [123]. P-selectin's ligands include PSGL-1 [116].

Integrins are responsible for the next step in T cell migration: firm adhesion of the cell to the endothelial lining [113]. Integrins are protein dimers, composed of an α-chain and a β-chain. Although 18α and 8β subunits have been discovered in mammals [124], the integrins involved in T cell migration are largely either in the β2 class – for example LFA-1 (leukocyte function-associated antigen-1; αLβ2 integrin), Mac-1 (αMβ2 integrin) or αdβ2 integrin, or α4 class – for example α4β7 and VLA-4 (very late antigen-4; α4β1 integrin) [124]. Crucial interactions include those of LFA-1 with its ligands ICAM-1 or ICAM-2 in the high endothelial venules, and α4 integrins binding to VCAM-1 or MadCAM-1 in blood vessels in inflamed tissue [113]. Integrins can also mediate rolling, under low-shear conditions [114].

Integrins on resting T cells are normally in an inactive, 'bent' formation. However, when the T cell becomes activated, the integrin receives signals from within the cell that cause it to acquire a new, extended formation that is able to bind ligands, albeit with only intermediate affinity. This is called 'inside-out' signalling [124, 125]. The integrin, on interaction with its ligand, can then alter its conformation again to become a high-affinity form ('open' conformation) [126]. When the integrin binds its ligand, the T cell comes to a stop, firmly bound to the endothelium.

The importance of the above ligand-receptor interactions to T cell migration and the consequent functioning of the immune system is underlined by the phenotypes of the individuals in whom they are defective. Problems with the early steps of T cell transmigration result in an immune disorder termed leukocyte adhesion deficiency (LAD), which can take several forms. LAD I results from impaired expression or function of the β-chain of the αβ integrins, such as LFA-1 [127]; this form of LAD results in moderate to severe immunodeficiency, as T cells and other leukocytes cannot mediate firm adhesion, a step required in order to transit through the blood vessels. LAD II results from a lack of fucosylated selectin ligands and leads to a less severe immunological deficit due to impaired leukocyte rolling [128]. A third type of LAD, LAD III, is a disorder whereby individuals have faulty inside-out signalling and hence cannot activate their integrins to reach a high-affinity state in response to signals [129]; patients with this rare disorder suffer recurrent infections and profound leukocytosis

[130]. The severity of these disorders indicates that successful leukocyte migration is necessary for the proper functioning of the immune system.

The final step in the process of transendothelial migration is diapedesis, in which the T cell passes through the endothelial monolayer lining the blood vessel [107]. Initially it was thought that cells could only pass through the junctions between endothelial cells (para-cellular transmigration); however, more recent studies have provided evidence suggesting that transcellular diapedesis – in other words, migration through the endothelial cells themselves – is a common phenomenon [131, 132]. However, before either para-cellular or trans-cellular migration can occur, migration along the endothelium must first take place in order to find sites permissive to the passage of the cells. Before initiating migration, therefore, T cells migrate along the surface of the endothelium [133] to find an appropriate site, which tends to be either a junction between endothelial cells or a region of the endothelial cell away from the nucleus: cells cannot migrate at the site of the nucleus [132]. The T cell is thought to extend podosomes to ‘probe’ the endothelium in order to locate an appropriate site [134]. Adhesion receptors are thought to be crucial for both para-cellular and trans-cellular migration to occur [135], particularly ICAM-1, VCAM-1 and LFA-1.

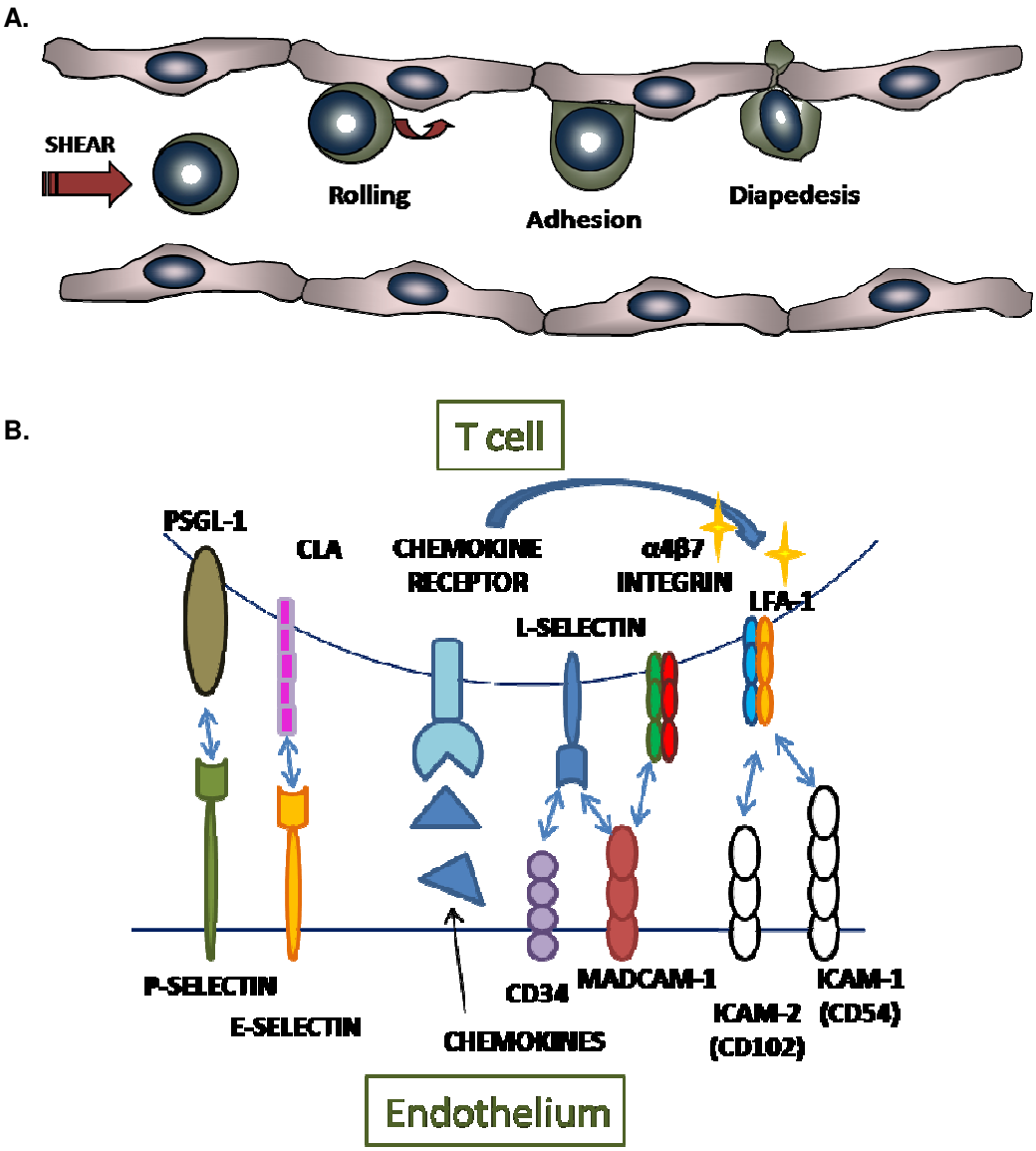


Figure 1.3. Transendothelial migration by T cells.

A. T cells travel through blood vessels under significant shear force. In order to exit the vessel and pass through the endothelium to the tissue, they follow a multistep process initiated with rolling along the endothelial surface, mediated by selectins. This is followed by firm adhesion, as a result of integrins interacting with their ligands. Finally, the T cell can pass through the junctions between endothelial cells or the endothelial cells themselves in a process called diapedesis, before travelling through the tissue. **B.** A selection of molecules involved in T cell migration. E-, P- and L-selectin mediate rolling; chemokine receptors, once bound by their specific chemokines, activate integrins to initiate firm adhesion.

Chemokines

Chemokines are molecules that help to direct the migration of T cells and other leukocytes to particular tissues. They and their receptors are named according to the pattern of cysteine residues in the chemokines: C, CC, CXC or CX3C [136], with the C standing for cysteine and the X for a different amino acid. There are around 50 different chemokines and 20 different receptors in humans [137], although not all these receptors are expressed by T cells. In addition to being classified according to their structure, they can be divided into inflammatory and lymphoid, or homeostatic, chemokine receptors [106, 138]; inflammatory chemokines direct cells to sites of inflammation, whereas lymphoid chemokines are constitutively present in lymphoid organs, maintaining normal lymphoid traffic [106, 138].

Chemokines are central to the activation of integrins during T cell (and other leukocyte) migration, between the rolling and firm adhesion steps [113] (see Figure 1.3B). They are tissue-specific molecules secreted by the endothelium, which bind specific receptors on the leukocyte. Table 1.2 shows a selection of chemokines, their receptors and the tissues to which they direct leukocyte homing. On binding of the chemokine to its surface receptor (outside-in signalling), signals within the cell direct the integrin to alter its conformation to a more high-affinity shape (inside-out signalling), so that it can bind strongly to its ligand and allow the T cell to adhere to the endothelium. In addition, after crossing the endothelial barrier, a chemokine gradient continues to direct the migrating T cell towards the site of inflammation [138].

Lymphoid chemokines include CCL19 and CCL21 [139], which bind the chemokine receptor CCR7; this is constitutively present on naïve T cells and on central memory cells [40, 138], allowing them to circulate via the lymph nodes. CXCR5 is also a lymph node-homing chemokine receptor, binding to the follicular-compartment-expressed chemokine CXCL13, but is expressed by a specialised subset of helper T cells, follicular B Th cells [138]; these provide help for antibody production by B cells [140]. CXCR4 is also highly-expressed on naïve cells, allowing entry to high endothelial venules (HEVs) and hence the lymph nodes, as well as the bone marrow [141].

Once primed, T cells upregulate chemokine receptors that allow them to migrate to specific tissues. Skin-homing T cells express a number of different chemokine receptors which co-operate to direct the cell to cutaneous tissue. Among the most prominent are CCR4, expressed by all CLA+ T cells [138] and CCR10 [142], the ligands for which have been found on both inflamed and uninflamed cutaneous

endothelium [143, 144]. CCR5 has been suggested to direct homing to the liver [145] and CCR9 to the gut, since its ligand CCL25 is expressed in the small intestine [146]. Chemokines have been implicated in a number of diseases. Multiple sclerosis (MS), a disease in which the myelin of the nervous system is destroyed by T and B cells, has been suggested to be abrogated by injection of neutralising antibodies against CCL3 [147]. Studies have also found an accumulation of monocytes bearing CCR5, a CCL3 receptor, and CCR1 in the CNS of MS patients [148]. CCR1 has been linked to a large number of inflammatory disorders, including MS, rheumatoid arthritis and kidney disease [137], and high levels of CCL3 compared to CCL4 have been implicated in the development of insulitis and diabetes [149]. Hence, chemokine antagonists are currently much-investigated as potential therapeutic tools [150].

Table 1.2. Chemokines and their receptors

Chemokine receptor	Ligand(s)	Associated tissue(s)	Cell type
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7, CCL13, CCL14, CCL15, CCL16, CCL23	CNS	monocytes, mast cells
CCR4	CCL17 (TARC), CCL22	skin	effector T cells, Tregs, mast cells
CCR5	CCL3 (MIP-1α), CCL4, CCL5 (RANTES), CCL8, CCL11, CCL14, CCL16	liver, CNS	effector T cells, monocytes, mast cells
CCR7	CCL19, CCL21	lymph node	naïve T cells, central memory T cells
CCR9	CCL25 (TECK)	gut	effector T cells, pDCs
CCR10	CCL27 (CTACK), CCL28	skin	effector T cells
CXCR4	CXCL12 (SDF-1α)	lymph nodes, bone marrow	naïve T cells, central memory T cells, mast cells

1.2 Peripheral regulation of the immune response

Central mechanisms exist to prevent the escape of autoreactive T cells generated in the thymus. Developing lymphocytes encounter MHC presenting self-antigen on the surface of cells in the medulla of the thymus and their fate is determined by the strength of their reaction to it: too strong and they are deleted, as they risk generating autoimmune responses [52, 151]. This mechanism is not fail-safe, however, and autoreactive T cells do occasionally escape the thymus to circulate in the periphery [152].

To protect against inappropriate activation, T cells are therefore inherently difficult to activate, as discussed in 1.1.1. Cell-intrinsic regulatory mechanisms include the upregulation of inhibitory receptors with increasing differentiation and the development of anergy if the correct activation signals are not received in full [58]. Apoptosis also plays an important role – for example, in the case of activation-induced cell death (AICD) [153, 154].

A further layer of regulation is provided in the periphery by a subset of CD4 T cells with immunosuppressive function: regulatory T cells, or Tregs. This prevents autoimmune responses from taking hold, and also guards against immunopathology generated as a result of valid immune responses continuing out of control [155, 156]. These cells are termed regulatory T cells, and can either exit the thymus as suppressive FOXP3+ cells [157-160] or are generated in the periphery. Conventional T cells can be induced to express FOXP3 [77] or to become other subsets of regulatory T cells that do not express FOXP3, such as Tr1 and Th3 cells [161, 162].

1.2.1 Cell-intrinsic peripheral regulation mechanisms

In order to prevent aberrant activation and damage to self tissue, responder T cells, both CD4+ and CD8+, have a number of in-built mechanisms to regulate their activation. First, if a T cell receives a TCR signal without associated costimulation, it may enter a state of anergy [58]. Anergic cells are unresponsive: they do not proliferate or release cytokines such as interleukin-2 (IL-2) [163], although addition of large quantities of this cytokine can break anergy induced *in vitro* or *in vivo* [164, 165]. An alternative to anergy is death. Previously-expanded T cells, when restimulated in the absence of costimulation, undergo activation-induced cell death (AICD) [153, 154]. Several different proteins can mediate AICD, including CD95L (Fas ligand). The receptor for CD95L, CD95, is expressed on all T cells; they upregulate CD95L after

activation. Binding of CD95L to its receptor causes caspase activation and apoptosis in the cell expressing CD95 [166]. TNF is also able to induce apoptosis after activation [167]. These mechanisms serve to limit the T cell response at its peak and to bring it to an end. There is an additional role for apoptosis in the peripheral regulation of T cell responses: that of death by neglect [168], or activated cell-autonomous death (ACAD). This is thought to result from cytokine deprivation or lack of costimulation, leading to low levels of the anti-apoptotic factor Bcl-2 and consequent apoptosis [169].

In humans, but not mice, activated T cells upregulate MHC class II on their surface. This allows them to present antigen to each other (a process called T:T presentation) [170, 171]. Although activated T cells have been reported to express B7 costimulatory molecules as well as MHC class II [170, 172], T:T presentation has been found to lead to anergy of responding T cells [170, 171]. This could be another way of ensuring that immune responses involving large infiltrates of activated T cells do not become uncontrolled and damaging.

Many costimulatory receptors present on the surface of naïve T cells are downregulated by more differentiated cells. For example, CD27 is lost after activation of CD4+ T cells, followed by CD28. (CD8+ T cells, however, downregulate CD28 first.) These two costimulatory receptors bind ligands expressed by the APC: CD28 binds B7.1 (CD80) and B7.2 (CD86) [59], whereas CD27 binds CD70 [173]. However, more-differentiated cells, which have lost these two receptors, can still receive costimulatory signals through other receptors such as OX40 and 4-1BB [174, 175].

As well as the downregulation of costimulatory receptors, there are addition cell-intrinsic checks on activation that become more prevalent as T cells differentiate. CTLA-4 is an inhibitory surface receptor which competes with CD28 for CD80 and CD86 binding [59], and has been reported to interact with the intracellular phosphatise SHP-2 [176], making it an effective inhibitory receptor. CTLA-4 expression has been shown to increase with age [177, 178], as the T cell pool in general becomes more differentiated. In addition, programmed death-1 (PD-1) expression increases in response to chronic stimulation, for example from persistent viruses [179], along with KLRG-1, another inhibitory receptor [102, 103]. These are likely to be extra safeguards against highly-differentiated, highly pro-inflammatory CD4 or cytotoxic CD8 T cells becoming activated inappropriately.

1.2.2 Regulatory T cells

Cell-extrinsic regulation provides another layer of peripheral regulation and is mediated by specialised subsets of T cells.

FOXP3+ natural Tregs

Identification

The existence of a regulatory subset of T cells was first postulated in the 1970s [180], initially termed 'suppressor cells'. In the absence of firm identification of these cells, however, they fell out of favour until 1995, when Sakaguchi and colleagues identified a subset of cells with suppressive function constitutively expressing the α-chain of the IL-2 receptor, CD25 [181]. This marker was originally thought to be expressed only by activated cells, but resting cells with the marker extracted from the peripheral blood of mice were shown to be anergic *in vitro* and to have suppressive function. These cells were subsequently shown to be present in humans as well [182, 183], although due to the more continuous spectrum of CD25 expression among peripheral blood T cells in humans, in this case they are specifically referred to as $CD25^{hi}$ cells [182].

FOXP3

FOXP3 is a transcription factor, the human orthologue of the murine protein Scurfin [184-186]. Mutations of the gene *Foxp3* in mice, where the resulting gene is non-functional, cause a systemic autoimmune disease known as scurfy; loss-of-function mutations of the human *FOXP3* gene are known to lead to a similar syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterised by lymphoproliferation, neonatal IDDM (insulin-dependent diabetes mellitus) and eczema, among other symptoms [184, 185]. FOXP3 expression was subsequently found to be crucial to Treg function [157-159].

One of the major characteristics of regulatory T cells, in addition to their ability to suppress activation of other cells, is their in vitro anergy [187]. FOXP3 is known to suppress transcription of the *Il-2* gene via interactions with NFAT (nuclear factor of activated T cells) [188] and in the same way to upregulate the inhibitory surface molecule CTLA-4 and the α-chain of the IL-2 receptor, CD25. It has also been postulated to interact with CREB and NF-κB [189], as well as AKT [190] to mediate Treg behaviour. Interactions between FOXP3 and AML/Runx1 have also been shown to inhibit IL-2 production and are potentially contributory to suppressive activity [191].

FOXP3 is the most reliable Treg marker discovered to date in humans. Its expression in CD4+ T cells in non-inflammatory environments correlates closely with suppressive function, more so than the original Treg identifier, CD25 [160, 192]. Although its expression seems entirely limited to Tregs in mice [157-159], FOXP3 has been shown to be upregulated by human conventional CD4+ T cells when they are stimulated *in vitro* [193]; most studies have found, however, that this does not confer suppressive capacity on the activated cells [194-196]. However, ectopic expression of FOXP3 confers suppressive capability on conventional, responder, CD25- T cells from both mice and humans [157, 158, 160, 197, 198].

Other natural Treg markers

Tregs have been described as having an 'activated' phenotype [199], constitutively expressing markers such as CTLA-4 [59, 160], GITR [200, 201] and HLA-DR[202], all normally seen only on activated CD4+ T cells (see Table 1.3) [203-205]. The search for a defining Treg marker is ongoing; many have been proposed, such as neuropilin-1 [206], and LAP [207-209], although the former is not Treg-specific in humans [210] and the latter is present specifically on a subset of Tregs, not the whole Treg population [207, 211]. The IL-7 receptor α chain, CD127, is expressed at a constitutively low level by Tregs, whereas responder CD4+ T cells express a reasonably high level [212, 213]; however, immediately upon activation, responder T cells transiently downregulate CD127 as well [214]. CD39 has also been suggested to identify Tregs [215, 216] but is also upregulated on activation [217].

All of the above, combined with the upregulation of FOXP3 by conventional T cells on activation, makes regulatory T cells difficult to identify in an inflammatory environment. However, more markers are continually being postulated; recently, it emerged that a promoter region of the *FOXP3* gene (referred to as the Treg-specific demethylated region, or TSDR) is demethylated specifically in FOXP3+ regulatory T cells [218]. It is methylated in conventional cells and remains so even when, on activation, they transiently upregulate expression of FOXP3. The TSDR therefore looks like a potentially useful marker for identifying true Tregs; however, in order to analyse methylation status, the cells must be purified, something not practical for many studies. For cells isolated from or investigated in a noninflammatory environment, FOXP3 remains by far the most reliable marker for Tregs [192].

Table 1.3. Treg markers

Marker	Expression	Similar expression on activated responders?	References
FOXP3	high	yes, transiently	[158, 192-194]
CD25	high (bright)	yes	[182, 183, 200]
CD39	constitutive (50-80%)	yes	[215-217]
CD127	low	yes	[212-214]
CTLA-4	constitutive	yes	[59, 204]
GITR	constitutive	yes	[200, 201, 205]
HLA-DR	constitutive	yes	[202, 203]
LAP (membrane-bound TGF-β)	activated subset	no	[207, 208, 211]
Neuropilin-1	constitutively high	yes	[206, 210]
TSDR	demethylated	no,methylated	[218]

Generation and maintenance

CD4+CD25^{hi}FOXP3+ 'natural' regulatory T cells [219] are known to be suppressive as soon as they exit the thymus as naïve cells [220, 221] and have been proposed to be self-reactive cells that escape deletion in the medulla [56, 222]. Notably, thymectomised, TCR-transgenic, RAG-deficient mice lack regulatory T cells altogether [158, 223], implying that TCR signalling in the thymus is crucial to their development.

When FOXP3+ cells initially exit the thymus, they express the long CD45 splice variant CD45RA. In cord blood, almost all Tregs are CD45RA+ [220], but in human adults 90% of Tregs have a memory (CD45RO+) phenotype [183, 224]. CD45RA+ regulatory T cells are, however, detectable in small numbers throughout adult life into old age [220, 221]. They are known to be suppressive and to express FOXP3, although at lower levels than CD45RO+ Tregs [225]. They may also potentially be used therapeutically, as they are expandable *in vitro* without losing either expression of FOXP3 or their suppressive capability – an attribute that is apparently lacking in their CD45RO+ Treg counterparts [226]. These expanded Tregs may then be transfused to block inappropriate immune activation, for example in response to transplants.

However, although Tregs are initially generated in the thymus, they may develop from other sources in the periphery [227, 228]. CD45RO+ Tregs in humans have been shown to divide very rapidly and to have short telomeres [228] and so without regular input of naïve cells from the thymus their numbers might be expected to decline rapidly. Tregs have nevertheless been shown to be stable in number, and even to accumulate, in aged individuals, whether humans or mice [229-231]. This observation has led to suggestions that they may be converted from peripheral, non-regulatory, CD4+ memory T cells [228]. A number of potential mechanisms for this conversion have been postulated by experiments performed *in vitro*, including via the influence of retinoic acid [232-234], TGF-β and IL-2 [77, 78, 235] and oestrogen [236].

The relative contribution *in vivo* of thymic compared to peripherally-converted Tregs is still a matter of debate. However, conversion of responder T cells to Tregs has been demonstrated *in vivo* under a number of conditions. Liang and co-workers [237] found that CD4+CD25- cells acquired CD25 and FOXP3 expression and a regulatory phenotype when transferred into normal, non-lymphopaenic mice, provided B7 was expressed in the recipients. Other studies have also reported induction of regulatory T cells *in vivo*, including experiments supporting the role of TGF-β [238], Treg induction as a result of expression of a G-protein receptor [239] and studies suggesting a role for

thymic exosomes [240]. Additionally, low quantities of peptide administered to mice induced a population of suppressive CD4+CD25+ regulatory T cells [241].

Function

Natural Tregs exert dominant tolerance over responder T cells – defined as tolerance which can be transferred into another host [242]. They suppress proliferation and production by responders of proinflammatory cytokines such as interferon-gamma (IFN-γ) and IL-2 [187, 243]. The precise mechanism by which natural Tregs exert their inhibition is still a matter of some debate. Their suppressive effects can be detected via *in vitro* suppression assays, where they require cell-cell contact with their target responder cells in order to suppress their activation, as transwell experiments have demonstrated [187, 244, 245].

The role of cytokines in Treg function is not entirely clear. Numerous studies have suggested that IL-10 and TGF-β are not required for suppression by Tregs *in vitro* [159, 187, 246] but a requirement for these cytokines cannot be entirely ruled out *in vivo*, following experiments performed in mice. Powrie and coworkers found that colitis, suppressed by regulatory T cells, was restored following injection of anti-TGF-β antibodies [247]. A more recent study by Ring et al showed that anti-IL-10 antibodies abrogated the ability of Tregs to suppress tissue inflammation [248]. Additionally, surface-bound TGF-β, whether in its active or inactive (LAP) form, has been implicated in FOXP3+ Treg-mediated suppression [207, 208, 211]. IL-35, a member of the IL-12 cytokine family, has also been suggested to play a role [249, 250]. Another proposed mechanism is that Tregs act to 'soak up' growth factors such as IL-2 [251], although why cell contact should be required for such a mechanism to function is not clear. It has, however, been suggested that Tregs may require proximity to their target cells rather than direct contact [250].

There is strong evidence to suggest that CTLA-4 is required for Treg function, perhaps even more than FOXP3 [252, 253], although even so it has not been universally found to be essential [254]. CTLA-4 has been suggested to be involved in Treg-mediated suppression of APC function by interacting with CD86 and CD80 on activated responder T cells or APCs [255] or even downregulation of these costimulatory molecules on the antigen-presenting cells [256, 257]. Tregs may also affect APCs by preventing their maturation [258]. They have even been suggested to lyse antigen-presenting cells [259] or to kill responders directly via perforin and granzyme [260]. It is probable that no single mechanism holds the key to Treg-mediated suppression: Tregs

are likely to control different immune responses, in different environments, in different ways.

Recent studies have implicated cyclic adenosine monophosphate (cAMP) and adenosine itself, both of which can have suppressive effects [215, 216, 261]. Pericellular adenosine is suggested to be generated by co-operation of the ectonucleases CD39 and CD73, expressed on the majority of Tregs in mice, and to function by binding inhibitory A2A receptors on the target cell [215, 216]. Cyclic adenosine monophosphate, known to be inhibitory, has been suggested to accumulate to high concentrations in the cytoplasm of Tregs. It is then suggested to be transferred to target cells through gap junctions formed between the tightly-apposed regulatory cell and responder [261].

Tregs are implicated in the pathogenesis of cancers: they are thought to suppress potential immune responses that could otherwise lead to rejection of the tumours [262]. As a result, controlling Treg function could be central to future cancer therapy. They are also being investigated as potential tools in the treatment of autoimmune diseases such as autoimmune type I diabetes[263], systemic lupus erythematosus (SLE) and also to prevent transplant rejection [264].

Induced Tregs

Two subsets of regulatory T cells which do not express FOXP3 are thought to be induced in the periphery, and are able to suppress via release of cytokines: Th3 cells and Tr1 cells.

Tr1 cells can be identified by their production of IL-10 [265]. They also express some TGF-β and high levels of IL-5, but very little IL-2 and IL-4, as well as being anergic *in vitro* [265]; in this respect they resemble natural Tregs. They do not, however, express FOXP3 [266]. They have been shown to be generated *in vitro* from both murine and human responder CD4+ T cells by means of repeated stimulation in the presence of IL-10 [265] and IFN-α [161] or via the action of immature dendritic cells [267]. *In vivo*, they can also clearly be generated peripherally: they can develop in response to oral administration of IL-10 and antigen in mice [268], and intranasal administration of peptides has also been shown to induce tolerant T cells that secrete IL-10, with no IL-2, also in mice [268]. Vitamin D3, in combination with dexamethasone, is also capable of generating Tr1 cells [269].

Tr1 cells have been shown to be important in the gut and to prevent colitis in mice [265] and IL-10 has been shown to improve intestinal inflammation in humans [270]. Interestingly, some Th1 cells have been shown to produce IL-10 and IFN-γ together and may play a role in gut inflammation [271-273].

Th3 cells are another subset of CD4+ T cell that are antigen specific, with similar T cell receptor clonality to Th1 cells, but produce large quantities of TGF-β1 [274]. They, like Tr1 cells, are generated peripherally during the course of the immune response [275] and can be induced by oral administration of antigen [276] or the administration of mucosal proteins [277]. These cells have also been shown to help alleviate colitis in lymphopenic mice [277] and TGF-β transgenic T cells can rescue CD25-knockout mice from potentially lethal autoimmunity [278], showing that TGF-β is capable of exerting strong suppression.

1.3 Ageing and the immune system

The human lifespan has increased dramatically in the last 150 years [279]. Throughout this lengthened lifespan, the immune system must be maintained to prevent the aged host from succumbing to infectious pathogens. Defects in various immune cell types have been reported in aged humans and animals, with the suggestion that even haematopoietic stem cells (HSCs), the precursors of all immune cells, may function less efficiently in older adults. In a model comparing the ability of old and young HSCs to repopulate the bone marrow of irradiated mice, older HSCs were outcompeted by the younger stem cells [280]. There is also a significant decline in the number of naïve B cells with age, along with an increase in memory B cells and autoreactive antibodies [281].

The innate immune system is also subject to changes with age, with an increase in proinflammatory cytokines such as IL-6 and TNF described in the elderly [282]. Innate immune cell subsets such as macrophages become less efficient; the total number of NK cells increases with age but their cytotoxicity declines [283]. The combination of these factors is thought to contribute to a generalised immune decline in aged humans, as demonstrated by their reduced response to vaccines compared with the young [284, 285].

1.3.1 Ageing and T cells

T cells are produced in the thymus, and a number of T cell-specific defects are known to be associated with age. Thymic tissue is completely functional in neonates, but with age this productive tissue is replaced by fat, a process termed ‘involution’; it is initiated by 1 year of age in humans [280]. Some functional tissue can still be found in the thymus even at advanced ages, but fewer T cells are able to mature in the involuted organ [47]. This leads to an accumulation of memory T cells in the periphery compared to naïve cells.

Naïve cells are not only present in lower numbers in old individuals compared to younger ones, but are also of lower quality than those in the young, showing lower responses to antigen [286, 287] and a decreased capacity to provide help [288, 289]. Additionally, a study by Haynes *et al* directly compared the function of memory cells generated from young and aged naïve cells. They found that memory cells generated from young naïve cells performed better, even a long period of time after their generation [290]. The lower fitness of haematopoietic stem cells in the aged may

contribute to this, although one study of bone marrow precursor cells have found no defect in the ability of these cells to generate functional T cells, whether sourced from young or old mice[291].

Memory T cells have shorter telomeres than their naïve precursors [292]. Although the telomere-lengthening enzyme telomerase is upregulated upon T cell activation [293], the cells gradually lose the ability to induce telomerase activity after repeated stimulation [93, 105, 294]. This can have a deleterious effect on the immune capacity of aged individuals as their more-differentiated memory T cells are unable to proliferate to the same extent as memory cells in the young [295, 296].

Additionally, persistent viruses such as cytomegalovirus (CMV) and varicella-zoster virus (VZV), which causes chickenpox and shingles, are found in a significant proportion of the adult population [297, 298]. During a human lifespan they are likely to reactivate several times [297, 298], often asymptomatically, but each time generating dedicated memory T cells. By advanced ages, individuals infected with CMV are thought to have a significantly less diverse, oligoclonal, T cell pool. This more limited range of T cell specificities reduces the breadth of possible immune responses and hence is thought to contribute to poor immune responses, particularly to new antigens, in the old [299, 300].

1.3.2 The immune risk phenotype

Elderly individuals infected with CMV have been found to have significantly lower life expectancy over two and four-year periods than CMV- individuals. For this reason, infection with CMV is one component of the ‘immune risk phenotype’ [301, 302], which predicts mortality. Other contributory factors include low numbers of B cells, a low CD4:CD8 T cell ratio and high levels of highly-differentiated CD8+ T cells [303], many of these characteristics resulting from CMV infection driving the T cell pool towards oligoclonality and senescence. These factors together may be expected to confer a significantly shorter life-expectancy on the individual affected.

1.4 Immunity in the skin

The skin is a large organ whose surface is largely exposed to the environment, making it essential to form an effective barrier against pathogens. It is also a highly accessible tissue that is often subject to inflammation, and can be induced to undergo acute inflammation by subcutaneous injection of antigen. Chronic inflammation within this organ can be monitored by studying individuals with inflammatory skin disorders, such as atopic dermatitis (AD). In this study, we investigated a potential role for memory and naïve Treg subsets in the pathogenesis of AD.

1.4.1 Skin immune surveillance

The barrier function of the skin is accomplished by means of a strong surface layer of keratin, generated by epidermal cells [304]. However, occasionally the skin barrier is breached, and to protect against this the skin is home to resident immune cells [305]; in addition, a large proportion of circulating memory T cells (around 30%) express the skin-homing adhesion molecule CLA [306], which facilitates their entry into this tissue. CCR8 has also been suggested as a skin-specific chemokine receptor [307].

The skin is a multilayered structure; underneath the barrier layer of keratin at the surface lie the epidermis and dermis (see Figure 1.4) [306], each monitored by different immune cells. The epidermis is monitored by Langerhans cells, specialised types of dendritic cell containing Birbeck granules, which are thought to be involved in engulfment [305, 308]. They can also be identified by their expression of Langerin, a molecule involved in ligand internalisation. Although fully competent antigen presenting cells, Langerhans cells also appear to have the ability to downregulate immune responses in certain situations; for example, they present skin self-antigen during the steady-state but do not initiate immune responses against self [308]. Keratinocytes are also important in immune surveillance, releasing inflammatory cytokines such as IL-1α and TNF [306], as well as chemokines to attract leukocytes. They can also express MHC class II [309], giving them the capacity to present antigen.

The dermis is largely composed of collagen, secreted by dermal fibroblasts [306]. It contains interstitial or dermal dendritic cells [23, 305, 310] which are also professional APCs, though less well-characterised than Langerhans cells. They express DC-SIGN and function slightly differently from Langerhans cells: Langerhans have been found to preferentially induce cytotoxic CD8+ T cells, whereas dermal DCs have been observed

to induce CD4+ T cells, skewing the immune response towards humoral immunity [310].

Both sets of dendritic cells, on activation, migrate through the dermal lymphatics to the draining lymph nodes of the skin, where they present antigen to T cells. Activation and consequent maturation of these antigen presenting cells also results in upregulated MHC class II, higher levels of costimulation and more efficient antigen processing [311, 312], to ensure optimal presentation of antigenic peptides to T cells in the lymph node and therefore efficient activation and migration of specific T cells to the skin itself. Additionally, antigen presentation has been suggested to take place in the skin itself during secondary immune responses, initiating T cell proliferation within the tissue [313].

A small number of memory T cells is also present even in noninflamed dermis [306], as a result of a very low level of E-selectin expression in dermal venules, and hence these cells can respond particularly quickly to a secondary cutaneous infection. FOXP3+ regulatory T cells have also been reported in noninflamed, healthy skin [314] and show a strong degree of expression of the skin-homing marker, CLA [315].

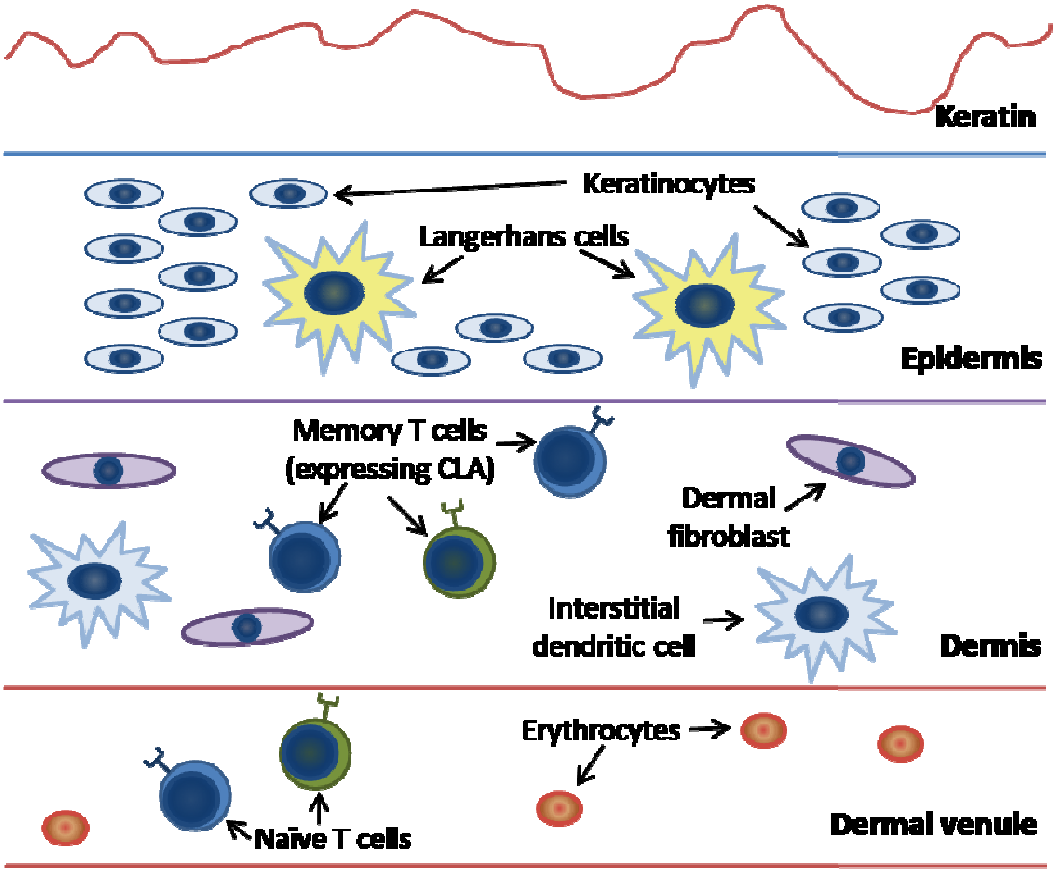


Figure 1.4. The structure and immunosurveillance of the skin.

Resident cutaneous immune cells include two different types of dendritic cell: in the epidermis, Langerhans cells, and in the dermis interstitial or dermal DCs. Keratinocytes in the epidermis also play an important role in cutaneous immunity, having the capacity both to present antigen and to release proinflammatory cytokines. Some CLA+ memory cells are able to traffic into uninflamed skin due to low but constitutive expression of E-selectin on dermal endothelium.

1.4.2 Monitoring cutaneous immune responses *in vivo*

It is possible to obtain a 'snapshot' of an ongoing cutaneous immune response by taking a biopsy of the affected area and cutting thin sections, which can then be stained with dyes or fluorescently-conjugated antibodies to highlight cells of interest. In order to monitor a secondary cutaneous response, an antigen to which the individual has previously been exposed (for example, PPD, a component of the BCG vaccine) is injected subcutaneously. A biopsy can be taken at a defined time after induction of the response.

However, our group has expanded the list of tools available to scientists investigating the *in vivo* cutaneous response by developing the skin blister technique [313]. Previously-encountered antigen, such as the tuberculin PPD, is injected under the skin to provoke a secondary immune response. At day 3 or day 7 after the injection, when the immune response is ongoing, a blister is formed over the inflamed site by the application of negative pressure. The blister is allowed to stay in place for 18 hours, during which time cells involved in the immune response pass into the blister fluid. The fluid, and cells within it, can then be harvested. This allows cells actively responding in an *in vivo* cutaneous immune response to be extracted and used in functional or flow cytometric analyses in the laboratory.

1.4.3 Atopic dermatitis

Atopic dermatitis is a hypersensitivity disorder also known as atopic eczema [316], which has been increasing in prevalence in the developed world [317, 318]. It has been reported in up to 20% of children [316, 319] but does not always persist into adulthood [318, 320]. It affects primarily the skin, causing widespread inflammation of the tissue, with associated itching and lichenification [320, 321]. It has been linked with other hypersensitivity disorders, such as asthma [322], although the nature of the link is controversial.

Many different factors are known to contribute to the risk of AD development, including environmental allergen exposure and infection, and there is also a strong genetic component: having one or both parents affected by AD is a major risk factor for the development of the disorder in their children [316]. Contributory factors are known to include defects in the skin barrier [323], including loss-of-function mutations of filaggrin [324], as well as immunologic factors. Langerhans cells and inflammatory myeloid DCs

have both been identified in AD lesions [321], TLR defects may also contribute [319] and T cells are thought to play an important role [324].

The acute phase of AD is thought to be primarily Th2-mediated, an allergic response involving eosinophils, mast cells and antibody responses [320]. There is also thought to be some contribution from Th17 cells [76, 325]: these are effector T cells that produce high levels of IL-17, promoting inflammation [326]. The chronic phase, however, is Th1-dominated[76, 321]. Adding a further level of complication, cytokines released from Th2 cells have also been shown to decrease the expression levels of filaggrin [324].

Tregs have long been of interest in relation to allergy development, and are implicated in the pathogenesis of atopic dermatitis in particular because a significant proportion of IPEX patients, who have mutated, non-functional FOXP3, show eczema-like symptoms [184, 185, 327]. However, as yet no conclusive evidence has been found for a Treg defect in atopic patients. Although some studies have reported a defect in suppression by atopic Tregs [328], or in their numbers in AD skin [329], this has not been a universal finding, with others finding equivalent suppression by AD Tregs and increased numbers of FOXP3+ cells in AD skin or peripheral blood [330-334]. However, in such a multifactorial disease and with the IPEX link, it remains probable that there is some Treg defect in at least a proportion of AD patients.

1.5 Aims of project

Although the regulatory T cell compartment as a whole is relatively well-characterised, little is known about the small naïve (CD45RA+) Treg subset and its contribution to the regulation of immunity throughout life. Additionally, we speculated that since atopic dermatitis is often a childhood condition from which individuals recover in adulthood, the naïve Treg pool may be defective in some patients with AD. The aims of this project were to investigate:

1. The heterogeneity of the Treg pool and changes throughout life.
2. The migratory patterns of naïve and memory Tregs.
3. The role of Tregs in *in vivo* immune responses in humans.
4. A potential role for different Treg subsets in atopic dermatitis.

Chapter 2. Materials and Methods

2.1 Obtaining human samples

This work was approved by the Ethics Committee of the Royal Free Hospital.

2.1.1 Healthy controls

Human peripheral blood was obtained from healthy laboratory staff or healthy volunteers aged between 20 and 91 years, with a male:female ratio of approximately 1:1. Blood was collected into syringes containing heparinized beads and processed immediately (see 2.3.2). Alternatively, peripheral blood was obtained in the form of buffy coats (National Blood Service, Colindale) and processed on delivery.

2.1.2 Patient samples

Peripheral blood samples from individuals with atopic dermatitis were obtained through the Department of Dermatology, Royal Free Hospital. Individuals were aged between 21 and 54 years with a male:female ratio of approximately 0.6:1.

2.1.3 Bone marrow samples

Samples of bone marrow and corresponding peripheral blood, from healthy individuals donating bone marrow for transplants, were obtained from the Department of Haematology, University College London.

2.1.4 Biopsies

5mm punch biopsies were taken, by a qualified dermatologist, from normal skin or from the site of inflammation in patients with atopic dermatitis. The surrounding skin was injected with 2% lignocaine/1:80,000 adrenaline local anaesthetic (Astra Pharmaceuticals Ltd, Kings Langley, UK) prior to the biopsy. The wound was closed with 4/0 Surgpro polypropylene suture (Tyco Healthcare UK Ltd, Gosport, UK). The skin was transported as quickly as possible to the lab in sterile saline.

Biopsies were mounted in Cryo-M-Bed (Bright Instrument Company Ltd, Huntingdon, UK) on cork disks, orientated so that the epidermis was perpendicular to the cork disk, and snap frozen in isopentane (Sigma-Aldrich, Gillingham, Dorset, UK) cooled in a bath of liquid nitrogen. The samples were then stored in a freezer at -80°C. 6µm frozen

sections were cut at -20°C using a Bright 5040 microtome (Bright Instrument Company Ltd, Huntingdon, UK) on to poly-L-lysine coated glass slides (Sigma-Aldrich, Gillingham, Dorset, UK). Poly-L-lysine coated slides were used in order to promote strong adhesion of the skin section to the slide. Two sections were mounted on to each slide. The sections were then left overnight to air-dry and fixed in fresh acetone for 10 minutes, followed by 99% ethanol for 10 minutes. The sections were air-dried for 10 minutes and then cling film-wrapped and stored until use in a freezer at -80°C.

2.1.5 Skin suction blisters

Skin suction has previously been shown to result in the formation of a split between the epidermis and dermis at the level of the lamina lucida. Suction blisters were induced by the application of negative pressure of 25-40kPa (200-300mmHg below atmospheric pressure) via a suction chamber (Medical Engineering, Royal Free Hospital, UK) centred over the site of testing for 2-4 hours using a clinical suction pump (VP25, Eschmann, Lancing, UK). Skin suction chambers with apertures of 15mm, 12.5mm and 10mm were used according to the size of the response and skin elasticity. In all cases, the size of suction chamber aperture selected ensured that the whole of the area of induration was sucked up in to the chamber with minimal incorporation of normal surrounding skin.

Suction was applied at warm room temperature (~22°C) until a unilocular blister measuring 10-15mm was formed over the site of the skin test. The blister was then protected overnight with a rigid adhesive dressing assembled using a Comfeel plus ulcer dressing (Coloplast, Peterborough, UK), a universal top (Sterilin, Fisher Scientific UK Ltd, Loughborough, UK), Micropore tape (3M healthcare, Loughborough, UK) and Tubigrip bandaging (Seton Healthcare Group plc, Oldham, UK).

Blister fluid was aspirated from the blister at 18-24 hours after induction using a sterile 23G needle and a 2ml syringe (Tyco Healthcare UK Ltd, Gosport, UK). The fluid was aspirated at the 18-24 hour time point in order to ensure maximal accumulation of cells within the blister fluid from the site of antigenic challenge in the skin. The recorded time of sampling was the time from skin injection to blister fluid aspiration. The volume of fluid recovered from the blister was recorded and suspended in 1.5ml conical tubes (Alpha Laboratories Ltd, Eastleigh, UK). The aspirated blister site was dressed with Betadine dry powder spray (Seton Healthcare Group plc, Oldham, UK) and a Mepore dressing (MoInlycke Health Care Ltd., Dunstable, UK). Volunteers were advised to

leave the dressing in place and to keep it dry for 24 hours before removing it and leaving the wound open to the air. The suction cups were dismantled after use and disinfected in Barrycidal 36 (Heraeus Instruments Ltd, Brentwood, Essex, UK) for a minimum of 24 hours.

The blister fluid was microcentrifuged at 650xg (3000 rpm) for 4 minutes (Microcentaur, MSE, Sanyo) to pellet the cellular contents. The supernatant was removed and aliquotted in to 1ml cryogenic tubes (Nunc, Thermofisher Scientific, Roskilde, Denmark) and stored at -70°C until analysed. The blister cell pellet was resuspended in 500µl of RPMI 1640(GIBCO, BRL Life Technologies, Paisley, UK) containing 10% human AB serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK).

2.2 Freezing Samples

2.2.1 Freezing lymphocytes for further live-cell analysis

In order to preserve living cells for further analysis, PBMCs were adjusted to 10-20x10^6/ml in pre-cooled foetal bovine serum (FBS) (Gibco Invitrogen Ltd, Paisley, UK). An equal volume of pre-cooled 20% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Ltd, Poole, UK) in FCS was added, to give a final suspension of 5-10x106/ml PBMCs in 10% DMSO/90% FCS. The suspension was then transferred into cooled cryotubes (Nalge Nunc International), 1ml per cryotube, and the cryotubes were placed in a chilled freezing container (Nalgene Cryo 1°C freezing container). The container was stored at -80°C overnight; the cryotubes were then transferred to storage containers and kept at -80°C until required.

2.2.2 Thawing lymphocytes

Frozen lymphocytes were thawed in an excess (10:1) of complete medium warmed to 37°C. They were immediately centrifuged at 1200rpm for 10 minutes and resuspended in complete medium for analysis.

2.3 Purification of Lymphocyte subsets

2.3.1 Peripheral blood mononuclear (PBMC) isolation

Blood samples were diluted with an equal volume of HBSS (Hanks' Balanced Salt Solution, Gibco Invitrogen) and the diluted blood was layered onto Ficoll Paque (GE Healthcare). 20ml diluted blood were layered onto 15ml Ficoll. The layered preparation was then centrifuged at 2000rpm for 20 minutes. PBMC at the interface were removed by aspiration and washed in HBSS twice: first at 1800rpm for 10 minutes and then at 1200rpm for 10 minutes, before being counted.

2.3.2 MACS isolation

This protocol allows the removal or purification of specific subpopulations of leukocytes by the addition of small, biodegradable magnetic beads attached to antibodies against particular cell surface markers (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells are incubated with these beads in a solution of MACS buffer (1x PBS, 2mM EDTA, 0.5% BSA). The buffer is degassed before use by placing a loosely-capped tube in a vacuum flask. After incubation, the cells are then passed through a column by a magnet: the labelled population of cells is immobilised in the column, while the unlabelled population passes through to be collected in suspension underneath. The immobilised, labelled population can, if required, be isolated by removing the column from the magnet and flushing it through with MACS buffer.

The following kits were used (the manufacturer's protocol was followed, except where indicated):

CD4+ microbeads [130-045-101]

CD4+ T cell isolation kit II [130-091-155]

CD4+CD25^{hi} Regulatory T Cell Isolation Kit, human [130-091-301]

The manufacturer's protocol was altered as follows: Cells were initially incubated for 10 minutes at 4°C with MACS buffer (95µl/10^7 cells) and non-CD4 biotin-conjugated antibody (5µl/10^7 cells). After this incubation, anti-biotin microbeads were added to the mixture (10µl/10^7 cells) and the sample incubated for a further 15 minutes at 4°C. Cells were passed through an LD column as instructed by the manufacturer. The run-through was washed and the cells counted, then incubated in a solution of MACS buffer (80µl/10^7 cells) with anti-CD25 beads from the kit (20µl/10^7 cells), for 15 minutes at 4°C. Cells were then run through an MS column as instructed by the manufacturer; the run-through was incubated with a second round of CD25+ microbeads at the same

concentration, before being run through a second MS column to obtain pure CD25- cells. The immobilised ($CD25^{hi}$) cells from the first MS column were flushed directly onto another MS column with 2ml MACS buffer and the solution allowed to run through. The cells immobilised on this second column were then flushed into a suitable container using a further 2ml MACS buffer.

CD45RA+ microbeads [130-045-901] and CD45RO+ microbeads [130-046-001]
These were used with the Regulatory T Cell Isolation Kit as an extra step between CD4 negative selection and $CD25^{hi}$ positive selection. Cells were incubated with the concentrations of beads indicated by the manufacturer, before being run through an LD column (hence, CD45RA beads were used to isolate CD45RA-CD45RO+ cells and CD45RO beads to isolate CD45RA+CD45RO- cells).

After isolation, cells were washed and resuspended in complete medium for further analysis or functional studies.

2.3.3 Cell-sorting

PBMCs were isolated as described in 2.3.1 and either total PBMCs or cell subsets (obtained via MACS as described in 2.3.2) were stained with fluorescently-conjugated antibodies against cell-specific markers (see 2.5.1). Cells were then suspended in RPMI containing 1% human serum and HEPES (Gibco, Invitrogen) and loaded into strainer cap-bearing FACS tubes (BD Falcon). Cells were sorted into serum-coated polyethylene tubes using a FACSAria (BD) and were immediately washed and resuspended in complete medium.

2.4 *In Vitro* Cell Culture

2.4.1 Standard cell culture conditions

Cells were cultured in 'complete medium': RPMI containing 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% human serum (all Sigma-Aldrich). They were cultured in 96-well round-bottomed plates (BD Falcon) at a total volume of 200µl/well in an incubator set to 37°C and 5% CO_2.

2.4.2 Measurement of cellular proliferation *in vitro*

Cells were stimulated using either T-cell Expander Beads coated with anti-CD3 and antiCD28 (Dynal) or plate-bound anti-CD3 (OKT3) in the presence of irradiated APCs, in either flat- or round-bottomed 96-well plates. OKT3 was used at a concentration of 1µg/ml. APCs were either total PBMCs or non-CD4 T cells and were irradiated by administration of X-rays to a dose of 40 Grays, then recounted and used at 1:1 with the cells being stimulated. After 72 hours' stimulation, 10µl of 0.0025MBq [^{3}H]-thymidine was added to each well and the plate was incubated for a further 16 hours. Cells were then harvested onto glass microfibre filter mats (Wallac, Finland) using a cell harvester (Cambridge Technology, Watertown, MA, USA) and counts of incorporated [^{3}H]-thymidine were determined using a betacounter (Perkin Elmer, MA, USA).

2.4.3 Suppression assays

CD4+, CD4+CD25^{hi} and CD4+CD25- T cells were isolated as described in 2.3.2. Culture conditions were as described in 2.4.2 and cell cultures were set up in the following combinations: CD4+ alone; CD4+CD25- alone; CD4+CD25- with CD4+CD25^{hi}; CD4+CD25- with further CD4+CD25-; and CD4+CD25^{hi} cells alone. Cells were stimulated with T-Cell Expander Beads in round-bottomed 96-well plates, at a concentration of one bead per responder cell. 50,000 of each cell type were used per well. To assess proliferation, the cultures were pulsed after 72 hours' incubation with [^{3}H]-thymidine, as described in 2.4.2. To assess cytokine secretion, responder cells were first stained with CFSE (see 2.5.5) to allow for discrimination between responders and Tregs. The cells were incubated in the combinations described above, with T Cell-Expander Beads, for 48 hours and then restimulated with 50ng/ml PMA and 250ng/ml ionomycin (both from Sigma-Aldrich, Gillingham, Dorset, UK). After three hours, Brefeldin A (BFA; Sigma-Aldrich, Gillingham, Dorset, UK) was added at a final concentration of 5µg/ml and the cultures incubated for a further three hours. The cells

were then stained for cytokine production following the intracellular staining protocol (see 2.5.2).

2.5 Flow Cytometry

Flow cytometric acquisition was performed by three different models of flow cytometer for these studies. Sorting was performed on the FACSAria, which contains three lasers (488nm, 635nm and 350nm) and is able to detect up to ten different colours. Simple flow cytometric acquisition was performed on either a FACSCalibur, which has two lasers (488nm and 6500-650nm) and can detect four colours, or a BD LSR 1, which has 3 lasers (325nm, 488nm, and 640nm) and can detect six colours. All machines are from Becton Dickinson (BD), Oxford. Analysis of acquired data was performed using either CellQuest software (BD) or FlowJo software (TreeStar, Inc, OR, USA).

2.5.1 Surface staining by direct immunofluorescence

Direct immunofluorescence involves the addition to cells of specific antibodies directly conjugated to fluorochromes; this is a single-step procedure and does not require the addition of further antibodies. Table 2.1 summarises the antibodies used in these studies, the optimal concentration of which was determined by preliminary titration. In most cases, four- or five-colour protocols were used, which allow the detection of four or five antigens simultaneously. This is possible because fluorochromes such as FITC and PE are excited at the same wavelength of light, but emit at different wavelengths, so all fluorochromes can be detected by the flow cytometer at the same time using filters and compensation controls.

For staining, the required cell sample was added to a 5ml FACS tube (BD Falcon) in a volume of 100µl. Antibodies were added at pre-titrated optimal concentrations and the sample was mixed and incubated at room temperature for 15 minutes. The cells were washed in PBSA (Phosphate Buffered Saline in 1% w/v bovine serum albumin and 0.02% sodium azide) and centrifuged at 1800rpm for 5 minutes, then fixed in 200µl of 2% paraformaldehyde (PF) in PBS (Phosphate Buffered Saline). Samples were stored at 4°C for no more than 24 hours prior to analysis. When conjugated fluorochromes, such as PE-Cy7, were used, the antibody-cell mixture was incubated on ice for 30 minutes rather than at room temperature in order to maintain the stability of the fluorochrome. Samples were then washed and fixed in PF as above. In all cases, samples were kept on ice while analysis was taking place.

Table 2.1. Antibodies used in flow cytometry

Type of staining	Antigen	Isotype	Clone	Fluorochrome
Membrane	CCR4	Mouse IgG1	1G1	PE
	CD4	Mouse IgG1	SK3	PerCP
	CD25	Mouse IgG1	ACT-1	PE
		Mouse IgG1	2A3	PE-Cy7
	CD31	Mouse IgG1	WM59	FITC & PE
	CD39	Mouse IgG1	A1	FITC
	CD45RA	Mouse IgG2b	HI100	FITC
		Mouse IgG1	L48	PE-Cy7
		Mouse IgG2b	MEM-56	APC
	CD45RO	Mouse IgG1	UCHL-1	APC
		Mouse IgG1	UCHL-1	PE
	CD69	Mouse IgG1	L78	PE
	CD73	Mouse IgG1	AD2	PE
	CLA	Rat IgM	HECA-452	FITC
	CXCR4	Mouse IgG2a	12G5	PE
	Isotype control	Mouse IgG1, IgG2a, IgG2b Rat IgM	-	FITC / PE / PE-Cy7 / PerCP / APC
Cytokine	IL-2	Mouse IgG1	5334	PE
	IL-4	Mouse IgG1	3010.211	PE
	IL-10	Rat IgG2a	JES3-19F1	APC
	IFN-γ	Mouse IgG1	B27	APC
	TNF-α	Mouse IgG1	6401.1111	FITC
Intracellular	CTLA-4	Mouse IgG2a	BN13	PE
	FOXP3	Mouse IgG1	3G3	APC
	Ki67	Mouse IgG1	B56	FITC

2.5.2 Surface staining for CTLA-4

CTLA-4 is a molecule that is largely present in the cytoplasm of resting cells and routinely cycles between the cytoplasm and cell surface membrane [59, 335]. Surface staining protocols for CTLA-4 must be altered as a result of this. Cells to be stained with CTLA-4 were initially resuspended in a total volume of 100µl; 10µl of anti-CTLA-4 were added and the samples incubated for 30 minutes at 37°C. They were then washed in PBSA, centrifuged for 5 minutes at 1800rpm and restored to a volume of 100µl. Other surface antibodies were added and the samples incubated on ice for 30 minutes, before either being washed as before and fixed in 2% PF, or stained for FOXP3 expression (see 2.5.4). In order to stain for total (intracellular and surface) CTLA-4 expression, surface staining with the other antibodies was done as in 2.5.1 and anti-CTLA-4 antibody was added at the same time as anti-FOXP3 in the FOXP3-staining protocol, described in 2.5.4.

2.5.3 Intracellular cytokine staining

In order to detect the production of cytokines by different cell subsets, stimulation (in order to initiate cytokine production) and blocking via BFA (to prevent cytokine release) were necessary as described in 2.4.3. After 3 hours' incubation with BFA, cells were centrifuged at 1800rpm for 5 minutes, supernatants removed and the cell samples resuspended in a volume of 100µl. They were surface-stained for 15 minutes at room-temperature, as described in 2.5.1. Before the washing step, 100µl of Caltag A solution (fixation medium; Caltag Medsystems, Bucks, UK) were added to each tube and the mixture vortexed for 15 seconds. Samples were incubated for a further 15 minutes at room temperature, then washed in excess PBSA at 1800rpm for 5 minutes. The supernatant was tipped off and the tubes dried, before 100µl Caltag B solution (permeabilisation medium) were added to each tube, along with pre-titrated volumes of fluorochrome-conjugated antibodies against the cytokines being investigated. The tubes were vortexed and incubated for a further 15 minutes, before being washed in PBSA again and resuspended in 200µl 2% PF for analysis, to be acquired within 24 hours.

2.5.4 FOXP3 staining

FOXP3 is a transcription factor, located within the nucleus of cells, and is therefore less accessible than cytoplasmic antigens such as recently-translated cytokines. A stronger fixation/permeabilisation protocol is therefore required to detect its expression. Staining was performed using the antibody and kit from Miltenyi Biotec. The same kit and

protocol were used to stain for Ki67, another nuclear protein. Up to 10^6 cells were resuspended in a total volume of 100µl. They were first surface-stained as described in 2.5.1, then washed in ice-cold PBS at 1800rpm for 5 minutes. Fix/Perm solution from the kit was made up, according to the manufacturer's instructions, and 1ml added to each sample. The tubes were incubated for 40 minutes at 4°C. The samples were then washed again in ice-cold PBS, dried and resuspended in 100µl 1x Permeabilisation Buffer. A previously-titrated optimal concentration of anti-FOXP3, Ki67 and/or CTLA-4 antibody was added and the cells incubated for a further 30 minutes at 4°C. The samples were washed in permeabilisation buffer and fixed in 200µl 2% PF for analysis. They were acquired on the flow cytometer within 24 hours of staining.

2.5.5 CFSE staining

CFSE (carboxyfluorescein succinimidyl ester; Molecular Probes, Inc) can be used to measure proliferation, by staining a population of cells and then acquiring them on a flow cytometer after stimulation; as the cells divide, the dye becomes progressively diluted and so the green signal detectable by FACS becomes weaker. In this study, CFSE was used both to follow cell divisions and also simply to mark populations of cells – for example, responder T cells, to distinguish them from the Tregs with which they were cultured.

The cell sample to be stained was centrifuged at 1800rpm for 5 minutes and the pellet resuspended in warm PBS. An equal volume of warmed, prediluted CFSE in PBS at a concentration of 1µM was added, to give a final concentration of 0.5µM, and the sample was mixed. The cells and dye were incubated for 13 minutes at 37°C. The reaction was stopped by adding FBS to a concentration of 10% and placing the sample on ice in the dark. After 10 minutes, the sample was washed at least twice in complete medium and then resuspended in complete medium at a suitable concentration for cell culture.

2.6 Anergy Induction

2.6.1 Generation of a PPD-specific CD4+ T cell line

PBMCs were harvested from the blood of volunteers with a strong PPD-specific response, and incubated with 1µg/ml PPD at 37℃ and 5% CO_2. After two weeks the cells were restimulated by adding PPD to PBMCs from the same donor, incubating for one hour, then irradiating (40Gy) and adding these cells to the culture, This was repeated every two weeks for six to eight weeks, after which the cells were assumed to be PPD-specific.

2.6.2 Induction of anergy

PPD-specific CD4+ T cells were incubated with plate-bound 0.05µg/ml, 0.1µg/ml or 0.5µgml OKT3 overnight (or in plates coated with PBS as a control). Cells were then removed from the plates, counted and washed to remove as much debris as possible before being phenotyped or used in functional assays.

2.7 Immunohistochemistry

In order to investigate the presence or absence of T cell populations in the skin, sections from biopsies (see 2.1.4) were stained using two layers of antibodies: first, antibodies specific for markers on the cells but not conjugated to any fluorochrome (Table 2.1). These were followed by fluorochrome-conjugated secondary antibodies, which were isotype- and species-specific anti-immunoglobulin. Alternatively, biotin-conjugated primary antibodies were used, followed by a fluorochrome (Cy3)-conjugated streptavidin second layer. Table II summarises the antibodies used to stain skin sections.

In order to stain the skin samples, slides bearing the sections were first removed from storage at -80°C and allowed to come to room temperature in a humidified environment. Slides were washed in 1x PBS for 5 mins, after which Protein Block was added (DakoCytomation UK Ltd, Ely, UK) and incubated for 20 minutes. The block was removed and primary antibodies added, diluted to pre-titrated optimum concentrations in 1x PBS. 40µl were added to each section. The slides and antibody were incubated at 4°C overnight. Slides were then washed for 10 minutes in the dark; this wash was repeated for a further 10 minutes, before the slides were dried and secondary antibodies (again diluted to optimum concentrations) were added at 40µl per section. The sections and antibody were incubated for 45 minutes at room temperature. The

slides were washed two further times, for 10 minutes each, and then dried. They were mounted using Vectashield with Dapi (Vector Laboratories, Burlingame, CA) and the stained sections were stored in the dark at -20°C prior to analysis. They were analysed using a Leica DMLB microscope with a 40x objective, in conjunction with a Cool SNAP-Pro cf Monochrome Media Cybernetics camera and ImagePro PLUS 6.2 software.

To count cells, the five largest infiltrates (or as many large infiltrates as possible if fewer than five were available) within each section were selected and positive cells within the field of view of each infiltrate were counted by two independent researchers. An average was taken for each section.

Table 2.2. Antibodies used in immunohistochemistry

Primary/secondary	Antigen	Isotype	Clone	Conjugate
Primary	CD4	Mouse IgG1	SK3	Unlabelled
		Rat IgG1	YNB46.1.8	Unlabelled
	CD8	Mouse IgG1	RPA-T8	Biotin
	CD45RA	Mouse IgG2b	HI100	FITC
	CD45RO	Mouse IgG2a	UCHL1	Unlabelled
	FOXP3	Rat IgG2a	PCH101	Biotin
	Ki67	Mouse IgG1	MIB-1	Unlabelled
Secondary	Anti-mouse Ig	Goat $F(ab')_2$ fragment	-	AlexaFluor 488
		Goat $F(ab')_2$ fragment	-	AlexaFluor 546
	Anti-rat Ig	Goat $F(ab')_2$ fragment	-	AlexaFluor 546
	Streptavidin	-	-	Cy3
	Anti-FITC	Goat IgG (polyclonal)	-	AlexaFluor 488

2.8 Migration studies

2.8.1 Growth and activation of endothelial cells

Dermal endothelial cells (HDMEC-c dermal microvascular endothelial cells from adult dermis; PromoCell, Germany) were cultured in 25cm^2 tissue culture flasks in endothelial cell growth medium (PromoCell). When confluent, the cells were removed from the flask with trypsin (PromoCell) and plated onto a flat-bottomed 96-well plate among as many wells as required (at least six per condition to be investigated). They were incubated at 37℃ and 5% CO_2 until confluent in each well. 24 hours before testing migration of T cells, the endothelial monolayers were stimulated with 100ng/ml TNF and 100U/ml IFN-γ.

2.8.2 Migration

The T cell subsets to be investigated were isolated the day before the migration assay using MACS technology and incubated overnight, without stimulation, at 37℃ and 5% CO_2. They were then resuspended in serum-free medium (AIM-V; Gibco, Invitrogen) at a concentration of 200,000 cells/ml. To perform the migration assays, endothelial monolayers were first washed with HBSS, then 100µl of cell suspension was added and incubated at 37℃ for 2-4 hours. Each condition was repeated 6 times. After the requisite time had passed, the plate was placed in a culture chamber (37℃, 5% CO_2) attached to a Zeiss Axiovert 200M light microscope. Time-lapse videos were obtained using a Hamamatsu Orca-ER digital camera and Simple PCI software, with images being taken every 10 seconds for 5 minutes. Cells which had migrated and were hence under the endothelial monolayer appeared darker and flatter; these were counted, along with the unmigrated cells in the field of view, so that the proportion of cells migrating after 2 or 4 hours could be estimated. The mean of six repeats was taken.

2.9 Statistics

Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, USA). Student's *t*-tests were used to analyse normally-distributed data; where data could not be assumed to be normally distributed, either the Wilcoxon Matched Pairs test or the Mann-Whitney test were used, depending on whether samples were paired or unpaired. Correlations were calculated using Spearman correlation calculations. P values are indicated on the majority of graphs with asterisks or text: ns (not significant), * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Chapter 3. Maintenance of human FOXP3+ Tregs throughout life

3.1 Introduction

In order to maintain closely-regulated immunity throughout life, the pool of Tregs must be sustained over the human lifespan of 8 decades. The majority of Tregs in adult humans have a highly-differentiated phenotype, with 90% expressing the memory marker CD45RO [224]. Tregs are generated in the thymus, as CD45RA+ cells, but numerous studies have demonstrated that they can also be generated in the periphery, following conversion of conventional CD4+ T cells into CD45RO+ FOXP3-expressing cells with an ability to suppress [77, 78, 232, 235, 236]. There is presently widespread debate about the proportion of Tregs in adult humans that have been generated thymically compared to the proportion converted peripherally from responder CD4 T cells, and it is not yet easy to identify which Tregs have been produced by either process.

This study aimed to compare the phenotypic and functional attributes of naïve Tregs with the more differentiated CD45RO+ subset in order to further define the interrelationship between the two subsets. Recently thymically-produced cells (recent thymic emigrants, or RTEs) have been shown to express CD31 in conjunction with CD45RA [69] and the prevalence of these cells within the Treg pool was investigated in the context of ageing. We also investigated another potential mechanism by which regulatory T cells could be generated from conventional CD4+CD25- cells in the periphery: anergy induction.

3.2 Changes in the Treg pool with age

3.2.1 The proportion of CD25^{hi} cells within the CD4+ T cell pool increases with age

In order to investigate changes in the Treg pool with ageing, samples of blood were taken from healthy volunteers of different ages and peripheral blood mononuclear cells (PBMCs) were stained for the Treg markers CD4 and CD25. As shown in Figure 3.1, no decrease with age was observed in the proportion of CD4+ cells that were CD25^{hi}, the classic Treg phenotype. In fact, a significant increase of these cells was seen during ageing (p=0.0132, r=0.3210, n=62).

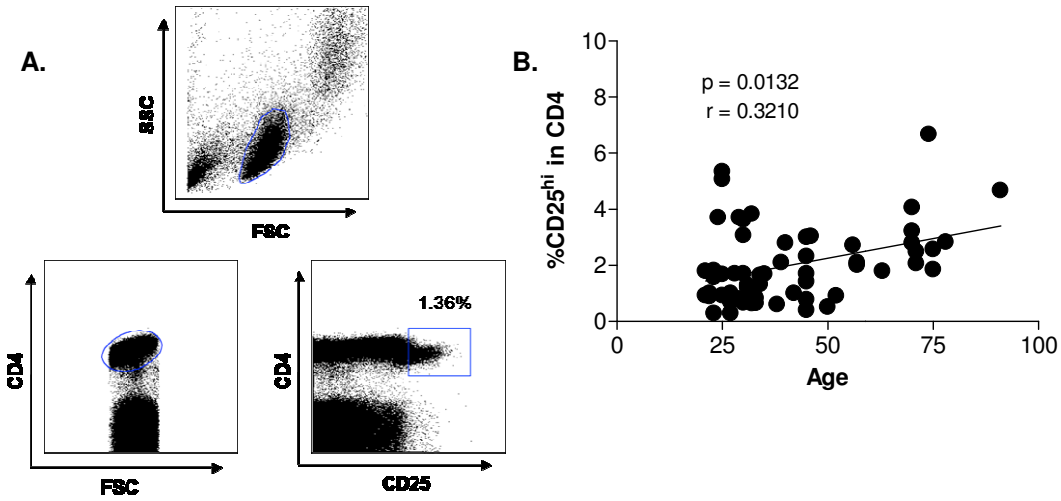


Figure 3.1. The proportion of CD4+ cells expressing CD25 to a high level increases with age.

Peripheral blood mononuclear cells were obtained from healthy volunteers aged between 20 and 91, and stained directly *ex vivo* for CD4 and CD25. Cells expressing the highest level of CD25 were gated and the proportion of the total CD4 compartment observed. **A.** Gating strategy for CD25^{hi} CD4+ cells. CD25^{hi} gate was set at the upper limit of CD25 expression in non-CD4+ lymphocytes. Percentages were then calculated after gating on CD4+ cells only. **B.** Graph showing changes in the proportion of CD4+ that is CD25^{hi} with age. (n=62)

3.2.1 Age-related alterations in proportions of Tregs expressing CD45RA and CD45RO

The composition of the conventional CD4+ T cell pool is known to change with age [64-68]. Although the Treg pool is maintained in older individuals, it is also likely to undergo changes with age in the differentiation status and sources of the cells which compose it. To investigate changes in the composition of the Treg pool with age, *ex vivo* Tregs from volunteers of various ages were stained with CD45RO and CD45RA to mark memory and naïve cells, respectively. A significant reduction with age in the proportion of Tregs bearing a CD45RA+CD45RO- phenotype was observed (Figure 3.2A). This was accompanied by a concordant increase in the proportion of Tregs (defined as CD4+CD25^{hi} cells) that was CD45RA- CD45RO+ (Figure 3.2B).

The line between 'naïve' and 'memory' is blurred with respect to T lymphocytes: CD45RA+, naïve T cells can become activated and begin to divide a few times before they lose expression of CD45RA [69, 336]. As a result, numerous groups have attempted to find a marker that is more specific for truly naïve T cells – i.e. which have recently exited the thymus and have not encountered cognate antigen. Recent studies have suggested that the adhesion molecule, CD31 (also known as PECAM-1, Platelet Endothelial Cell Adhesion Marker-1) may, when expressed on CD45RA-bearing cells, identify these RTEs: CD45RA+CD31+ CD4 T cells have been found to have longer telomeres, more TRECS and functions indicative of a less-differentiated phenotype than CD45RA+CD31- CD4+ cells [69, 70].

The proportion of Treg cells with an RTE phenotype and the proportion of CD45RA+ Tregs expressing CD31 were therefore also investigated. Although the proportion of RTE cells within the total Treg pool was reduced with age, the proportion of CD45RA+ Tregs expressing CD31 did not change significantly (Figure 3.2D), although there was a trend towards a decrease in the proportion of this subset. The continued presence of a significant proportion of CD31+ naïve Tregs in most donors, even in old donors, suggests that a small proportion of Tregs are likely to come directly from the thymus throughout life. This raises the possibility that thymically-derived Tregs may support the Treg pool throughout adult life, perhaps by extensive division.

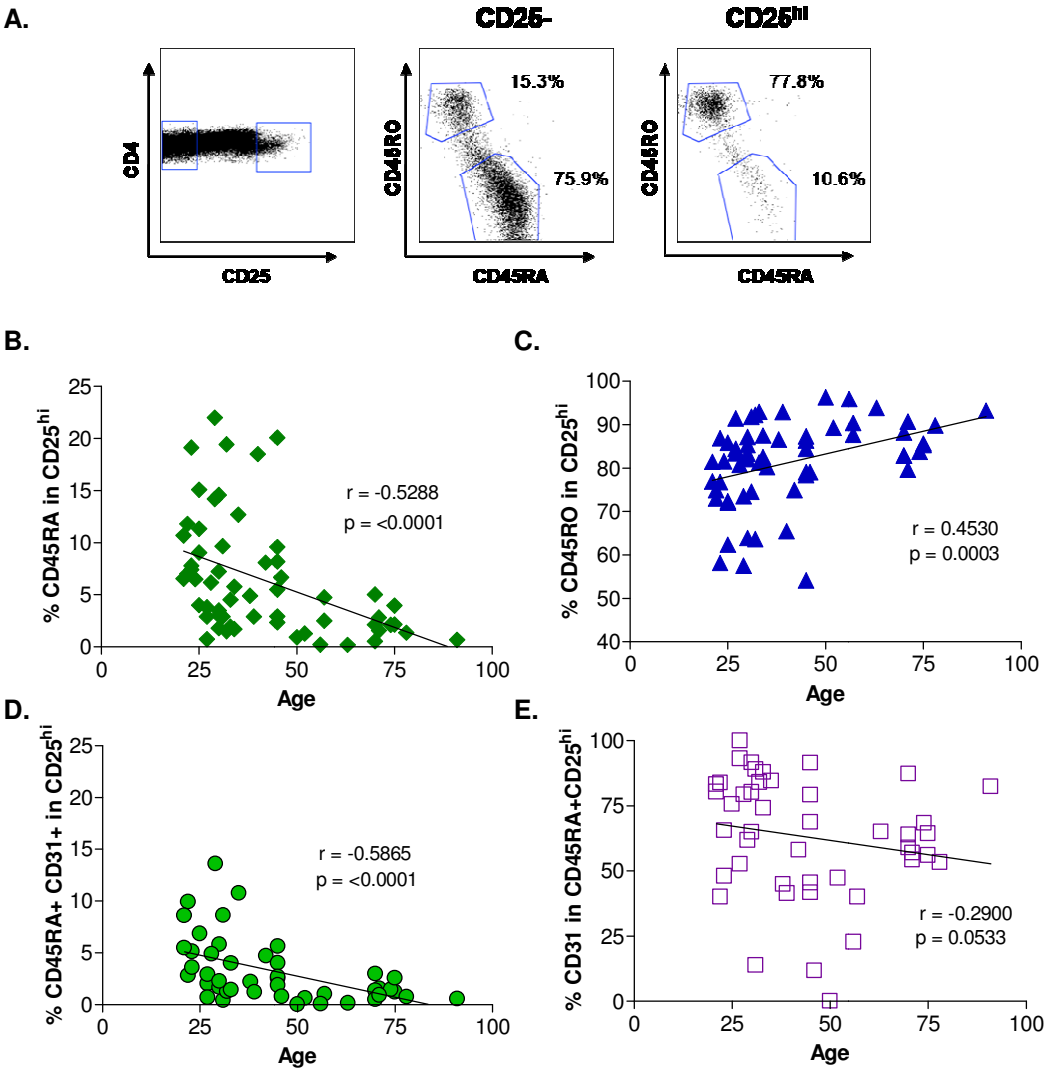


Figure 3.2. Changes in the composition of the Treg compartment with age

PBMCs from healthy volunteers (age range 20-91) were stained directly ex vivo for the Treg markers CD25 and CD4 as well as CD45RA, CD45RO and CD31, as described in Materials and Methods. **A.** Gating strateg for CD45RA+ and CD45RO+ CD25^{hi} and CD25- cells. **B.** Changes with age in the proportion of Tregs with a CD45RA+CD45RO- phenotype. (n=59) **C.** Changes with age in the proportion of Tregs with a CD45RA-CD45RO+ phenotype. (n=59) **D.** Age-associated changes in the proportion of regulatory T cells with an RTE (CD45RA+CD31+) phenotype. (n=57) **E.** Changes with age in the proportion of CD45RA+ Tregs expressing CD31. (n=57)

3.3 Naïve and memory Tregs proliferate at different rates

Previous work by our group demonstrated that human Tregs have a very high rate of turnover *in vivo* as well as short telomeres and susceptibility to apoptosis [228]. Naïve Tregs, however, are less differentiated and have much longer telomeres, equivalent to telomere lengths in naïve responder T cells [221]. If CD45RA+ Tregs also proliferate at a high rate, they may be able to support Treg numbers, even during later life after thymic involution. We therefore extended our earlier work to investigate the proliferative rates of the naïve and memory subsets within the Treg pool. Our previous studies used deuterated glucose (6,6-D-glucose), following its incorporation into and loss from the DNA of dividing cells. The small size of the naïve subset prevented us from using the deuterated glucose method in this case. Instead, we used a flow cytometry-based method, staining cells with fluorochrome-conjugated antibodies against the nuclear marker of proliferation, Ki67. This protein is expressed in the nucleus at all stages of the active cell cycle [337, 338].

3.3.1 Proliferation among CD45RO+ and CD45RA+ Tregs

As shown in Figure 3.3A, Ki67 expression was found to be highly prevalent among CD45RO+ Tregs, with 18%, on average, expressing the marker (mean 18.04%±0.99, n=37). Among CD45RA+ Tregs, the average expression was significantly lower than among the memory Treg subset, but still relatively high (mean 7.25%±1.48, n=37; $p<0.0001$, paired *t*-test). Responder (CD25-) CD4 T cells, however, had a much lower degree of proliferation than regulatory T cells, with Ki67 expression by 2.65% (±0.19) and 0.41% (±0.07) of memory and naïve responders, respectively. This confirms our earlier observations of a high degree of constitutive proliferation among resting Tregs, and extends them to show a high rate of turnover among CD45RA+, naïve, Tregs – higher than would be expected from a naïve T cell population.

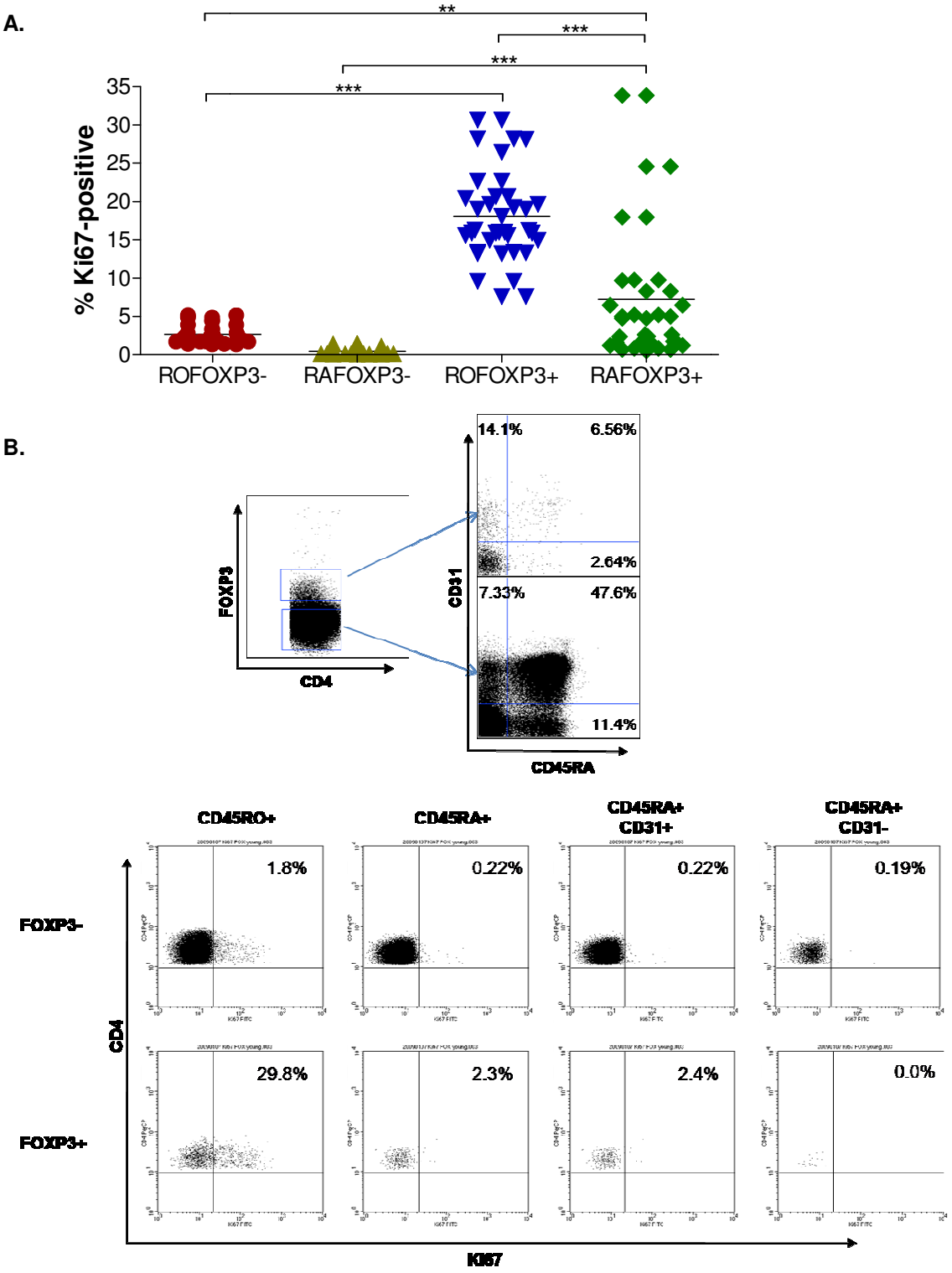


Figure 3.3. Proliferation of CD45RO+ and CD45RA+ CD4 T cell subsets

PBMCs from 37 healthy controls (age range 22-78) were stained *ex vivo* for CD4, CD45RO, CD31, Ki67 and FOXP3 using the FOXP3 staining protocol (see Materials and Methods). Naïve and memory cells were differentiated by expression of CD45RO: CD45RO- cells were assumed to be CD45RA+. **A.** Cumulative expression of Ki67 by CD45RA+ and CD45RO+ responders and Tregs. **B.** *Top:* Gating strategy for FOXP3+ and FOXP3- subsets. *Bottom:* Representative staining of Ki67 in subsets of Tregs and responders.

3.3.2 Proliferation among Treg and responder T cells is consistent over time

In order to be sure that this high rate of proliferation among Tregs was not due to an isolated event, but was consistently present, three donors were followed for a period of up to 18 weeks. Samples of blood were taken at three different time-points and their PBMCs stained for expression of Ki67. Figure 3.4 shows that the proportion of cells expressing Ki67 did not change in any subset, demonstrating that a large proportion of memory Tregs is dividing at any given time.

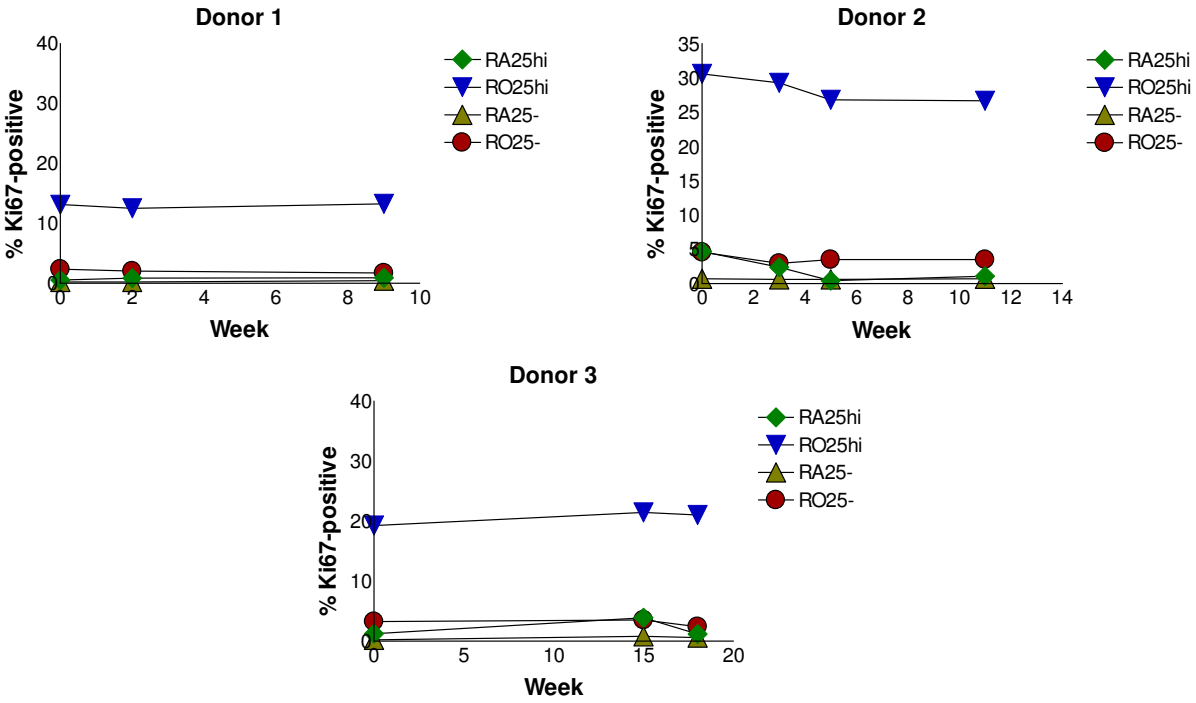


Figure 3.4. Proliferation of T cell subsets in peripheral blood is consistent over time.

Peripheral blood was processed on at least three separate occasions each from three healthy donors (donor 1: Weeks 0, 2 and 9; donor 2: weeks 0, 3, 5 and 11; donor 3: weeks 0, 15 and 18) and PBMCs stained for expression of Ki67, as outlined above. The graphs show expression of Ki67 by each CD4+ T cell subset at each time-point.

3.2.3 Proliferation within the naïve subset

CD45RA+ T cells can be divided into CD31+ RTEs and CD31- cells. We therefore investigated the rate of proliferation in these two subsets, both among the FOXP3- responder pool and FOXP3+ Tregs. Surprisingly, when data from all donors (aged 22-78) was investigated, a higher proportion of RTE Tregs expressed Ki67 than did their CD31- counterparts (see Figure 3.5A), with a mean of 2.87%±0.53 of CD31+ cells expressing the marker, compared to 1.54%±0.36 among the CD31- subset (n=18; p=0.0427; paired *t*-test). There was also a greater degree of proliferation among CD31+ than CD31- responder naïve cells. When the data from the old (>70) and young (<35) donors were analysed separately, it was found that a high proportion of the old donors' RTE Tregs expressed Ki67 (mean 4.08%±0.86), whereas among young donors the proportion was lower (mean 1.65%±0.28; p=0.0162; Figure 3.5B). This is likely to be the cause of the high RTE Treg turnover observed in the total cohort. On comparing the size of the naïve pools with the turnover within each pool, it became apparent that there was a greater degree of proliferation by naïve and RTE Tregs in donors with smaller pools of these subsets (Figure 3.5C), which is in line with previous observations that smaller pools of naïve T cells contain a higher proportion of proliferating cells [339], probably as a mechanism to maintain their numbers.

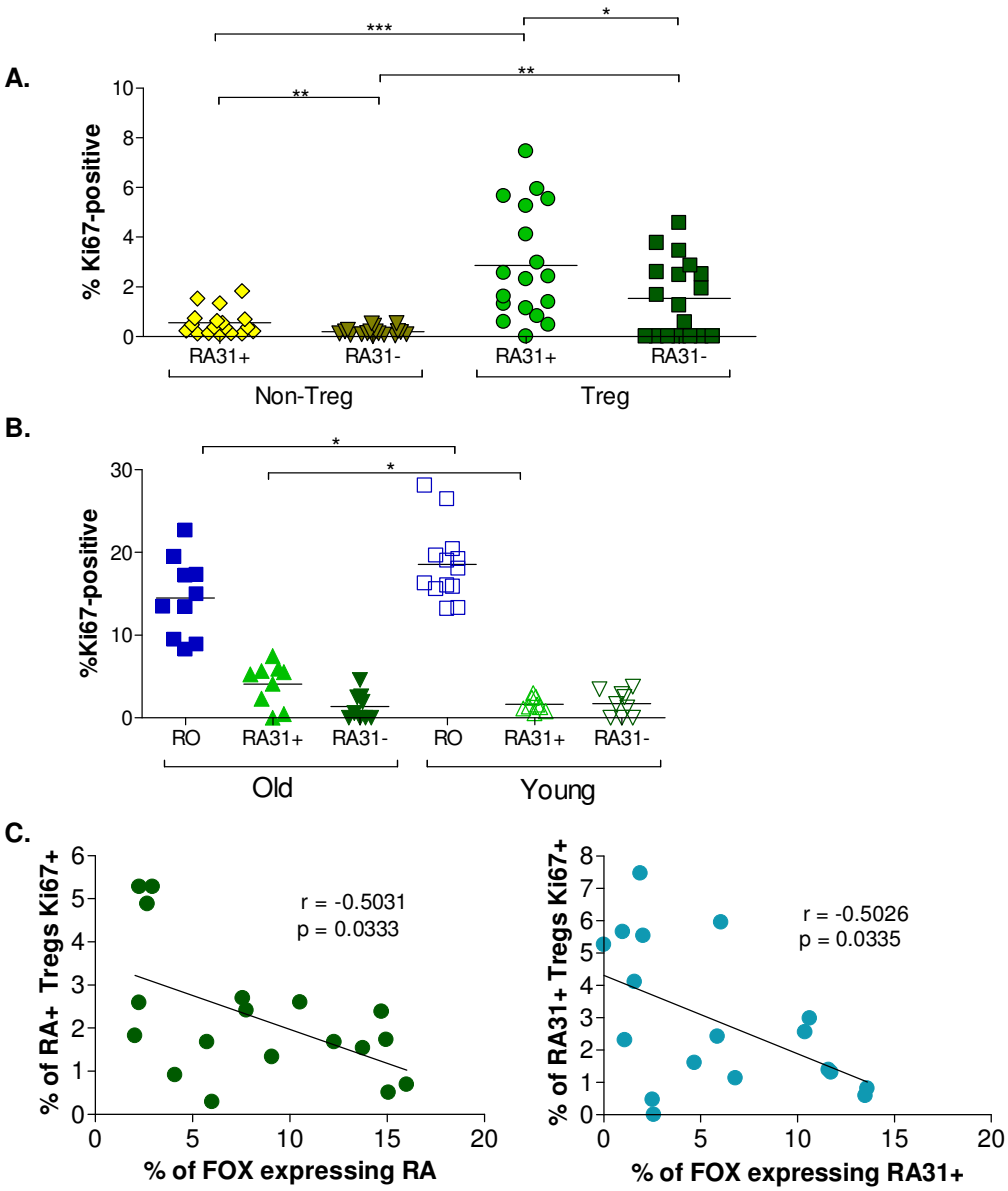


Figure 3.5. Proliferation of subsets within the CD45RA+ pool in old and young

PBMCs from 18 donors aged 22-78 were stained *ex vivo* as described above, with the addition of antibody specific for the RTE marker CD31. **A.** Expression of Ki67 by the CD45RA+CD31+ and CD45RA+CD31- subsets of both Tregs and responder (FOXP3-) T cells in all donors (n=23 for RO, 18 for RA subsets). **B.** Ki67 expression in all Treg subsets (CD45RO+, CD45RA+CD31+ and CD45RA+CD31-) in old donors (>70 years of age; n=10 for RO, 9 for RA subsets) compared with young donors (<35 years of age; n=13 for RO, 9 for RA subsets) **C.** Graph comparing size of CD45RA+ pool within the FOXP3+ population and percentage of CD45RA+ expressing Ki67. (n=18) *Right:* Graph comparing size of RTE pool within the FOXP3+ population and percentage of RTE Tregs expressing Ki67. (n=18).

3.3.3 Stimulation-induced proliferation of naïve Tregs drives them into the memory pool

CD45RA+ Tregs express a lower degree of Ki67 than CD45RO+ Tregs but are nevertheless proliferating. We hypothesised that on proliferation the CD45RA+FOXP3+ cells were being driven into the CD45RO+ pool. To investigate the rate at which naïve Tregs downregulated CD45RA and upregulated CD45RO, CD45RA+ cells, both Tregs and responder T cells, were sorted using a fluorescent cell-sorter and stimulated using anti-CD3- and anti-CD28-coated beads and their phenotypes evaluated at intervals. On proliferation, CD45RA+ Tregs rapidly downregulated expression of CD45RA and were almost universally CD45RO+ by day 4 (Figure 3.6A and B). Cells were confirmed to be dividing by the presence of large numbers of blast cells, as seen on forward scatter plots obtained via flow cytometry (not shown). This suggests that the lower rate of proliferation seen *ex vivo* among CD45RA+ compared to CD45RO+ Tregs may be explained, at least partially, by the fact that on dividing, CD45RA+ Tregs convert to show a memory, CD45RO+ phenotype. This observation confirms that CD45RO+ Tregs are likely to be generated from the CD45RA+ Treg pool by proliferation.

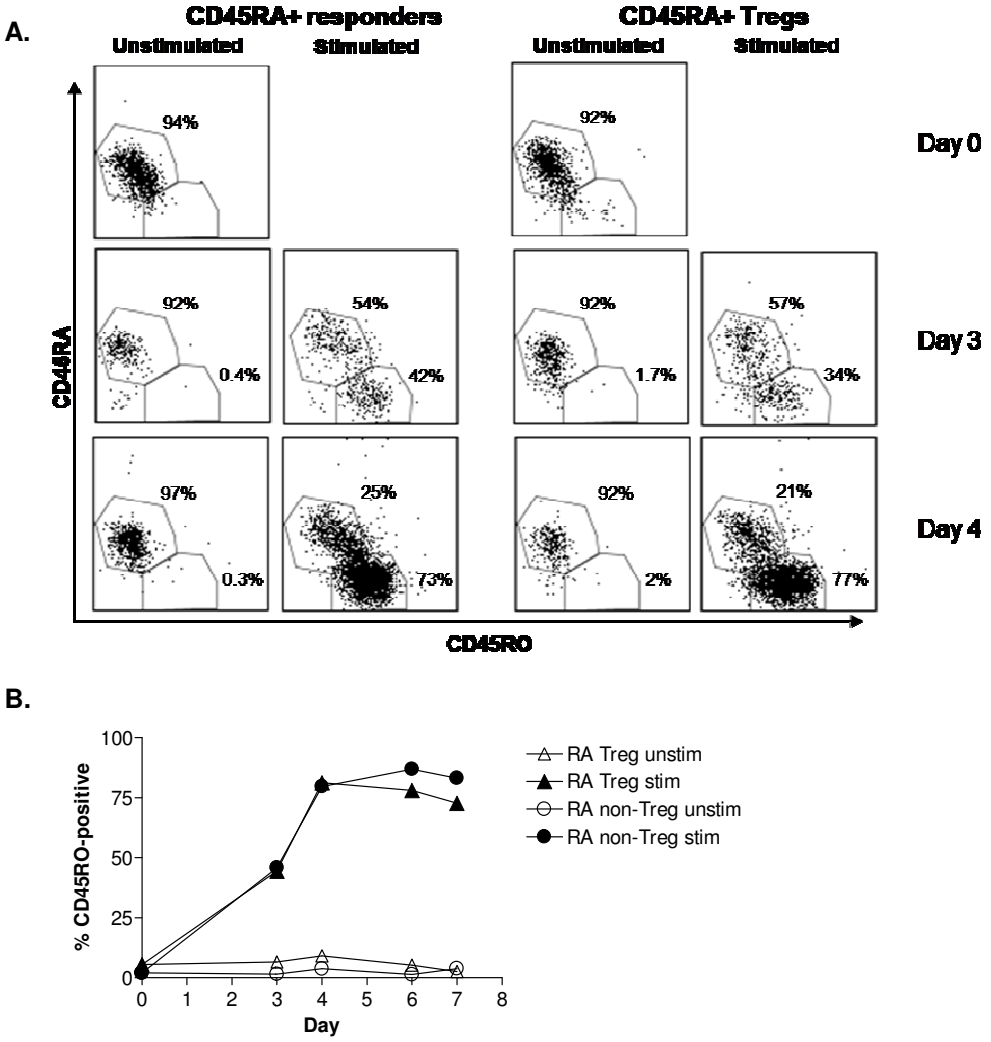


Figure 3.6. CD45RA+ Tregs acquire expression of CD45RO upon stimulation

CD45RA+ Tregs and responder CD4 T cells were isolated using MACS technology and cultured for up to 7 days in the presence of anti-CD3/anti-CD28 coated beads (0.1 beads/cell). Samples were taken at intervals throughout the culture period and stained for expression of CD45RA and CD45RO. **A.** Representative dotplots showing CD45RA/RO expression by Tregs and responders on days 0, 3 and 4 of stimulation, as well as unstimulated cells cultured over the same period. **B.** Graph showing acquisition of CD45RO expression by CD45RA+ Tregs and responder CD4 T cells.

3.4 Phenotype and function of naïve and memory Tregs

Some differences in phenotype between naïve and memory Tregs have already been elucidated – for example, the former are known to have lower expression of CD25 and some chemokine receptors [221]. This study aimed to expand upon the known differences between the two subsets, focussing particularly on molecules suspected to be involved in Treg function. The ectonucleases CD39 and CD73 have been postulated to cooperate to produce suppressive pericellular adenosine [215, 216] and CTLA-4 has been suggested to function in a number of ways to mediate suppression [59, 255, 257].

3.4.1 Differences in surface phenotype between naïve and memory Tregs

CD39 and CD73 have recently been strongly suggested to be involved in the function of murine Tregs [215, 216], via breakdown of ATP to adenosine, which then binds inhibitory A2A receptors on the target cell. We investigated the expression of these proteins on the CD45RA+ and CD45RO+ human Treg subsets, analysing expression levels via flow cytometry. We found a surprisingly low degree of expression of CD73 on human Tregs (mean 6.23%±0.93), and also very little expression on responder CD4+ T cells (mean 13.9%±2.2). Higher proportions of cells within the total lymphocyte gate expressed the marker, and with higher levels of expression (Figure 3.7A), indicating that the expression of CD73 is greater on lymphocyte types other than T cells (mean 24.8%±3.2; $p=0.0009$ compared with CD4+FOXP3+ cells, $p=0.0075$ compared with CD4+FOXP3- cells; n=6).

CD39, however, was expressed by a high proportion of human Tregs, as has been previously reported ([215, 216]; Figure 3.7B), with a mean of 60.6%±4.0 expressing the marker (n=17). Expression by responder CD4 T cells was highly-significantly lower than Tregs' expression (mean 5.83%±1.2; $p<0.0001$). On investigating CD39 expression by CD45RO+ and CD45RA+ subsets within Tregs and responders, we found a significantly smaller proportion of naïve than memory cells expressed the marker (Figure 3.7C). A mean of 79.5%±1.715 stained positive for CD39, but only a small minority of CD45RA+ Tregs did so (mean 12.8%±2.8; $p<0.0001$; n=8). These data may be explained by the fact that CD45RA+ Tregs are less likely to have been activated: CD39 is a T cell activation marker [217].

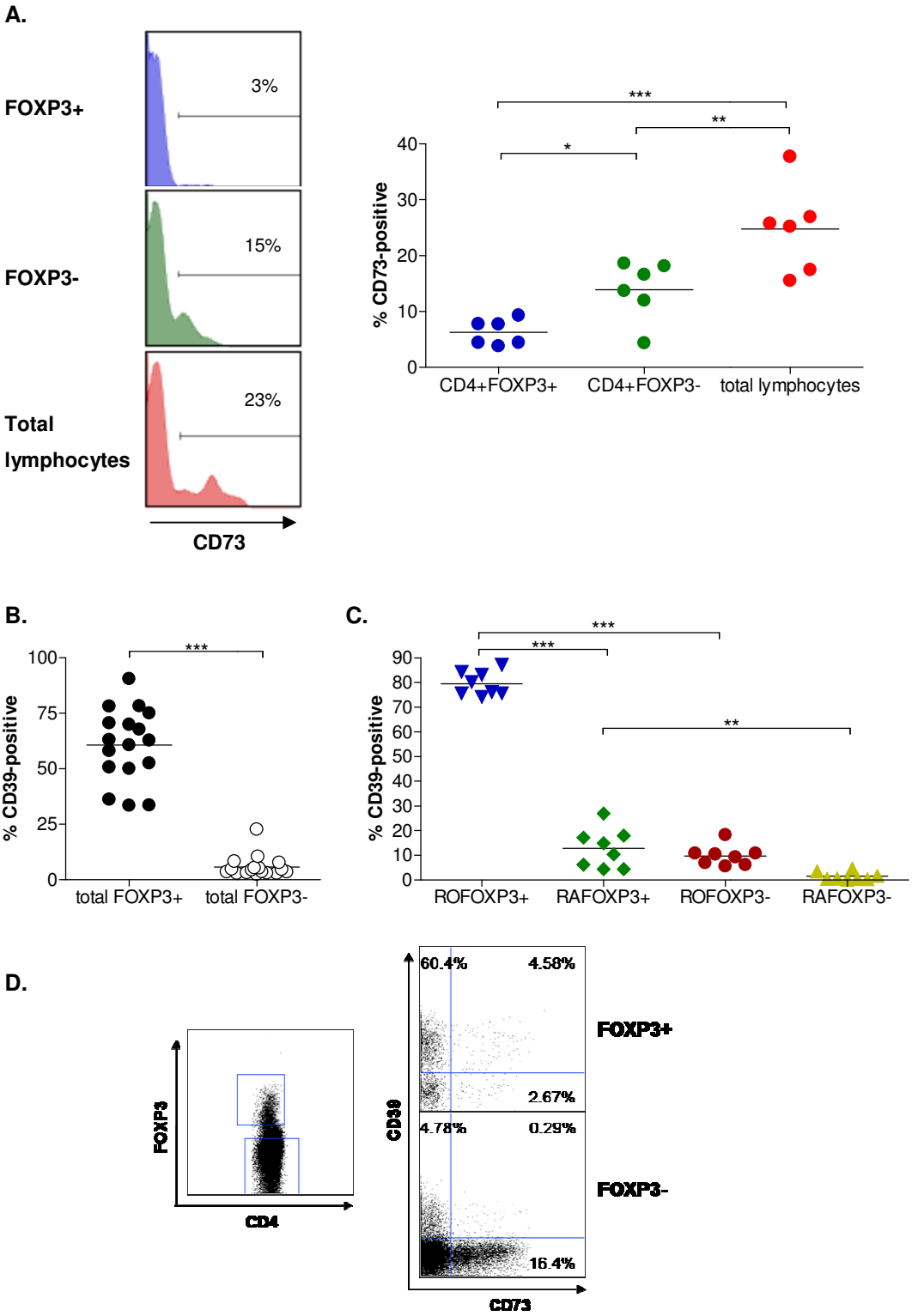


Figure 3.7. Surface expression of CD39 and CD73 on human CD4 Tregs and responder cells

PBMCs were stained *ex vivo* from healthy donors aged between 21 and 86. **A.** *Left:* Representative histograms showing CD73 expression by CD4+ T cells (FOXP3+ and FOXP3-) and total lymphocytes. *Right:* Cumulative data. (n=6) **B.** CD39 expression by CD4+ FOXP3+ cells and CD4+ FOXP3- cells. (n=17) **C.** CD39 expression by CD45RO+ and CD45RA+ subsets. (n=8) **D.** Representative staining showing CD39 and CD73 coexpression on FOXP3+ and FOXP3- CD4+ T cells.

3.4.2 CTLA-4 expression differs between naïve and memory Tregs

Another molecule implicated in the mechanism of Treg suppression is CTLA-4 [59, 255, 257]. This molecule is largely expressed intracellularly, cycling between the cytoplasm and the cell surface [59, 335]; as a result, two distinct protocols were used for staining: surface staining at 37℃, so that fluorescently-conjugated antibodies could capture and retain the molecule at the surface, and intracellular staining, by adding CTLA-4 at the same time as the FOXP3 antibody. Figure 3.8 shows representative staining of CTLA-4 and CD39 coexpression on FOXP3+ and FOXP3- cells; the majority of CTLA-4+ Tregs coexpress CD39, but only a small proportion of CD39+FOXP3+ cells expresses CTLA-4. Figure 3.9 shows that while a relatively high proportion of resting CD45RO+ Tregs expressed CTLA-4, both at the surface (mean 3.93%±1.0, n=6) and in total (mean 12.4%±1.2, n=7), significantly fewer CD45RA+ Tregs expressed the marker (surface mean 0.617%±0.31, p=0.0153; total mean 4.22%±1.6, p=0.0120). This is a not-dissimilar degree of expression to that seen among CD45RO+ responder T cells (surface mean 1.26%±0.29, p=0.0567; total mean 0.846%±0.18, p=0.0675). Again, CTLA-4 is known to be upregulated on activation of T cells [59, 204] and so these data may again be explained by the fact that CD45RA+ Tregs are unlikely to have been activated. Equally, however, CD39 and CTLA-4 may not be involved in suppression mediated by CD45RA+ Tregs.

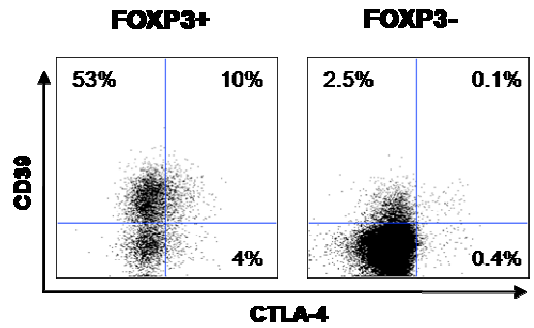


Figure 3.8. CD39 and CTLA-4 coexpression by human CD4 Tregs and responder T cells.

Representative staining of CD39 and CTLA-4 on *ex vivo* human T cells gated on CD4 and FOXP3 expression. Results are representative of at least four donors.

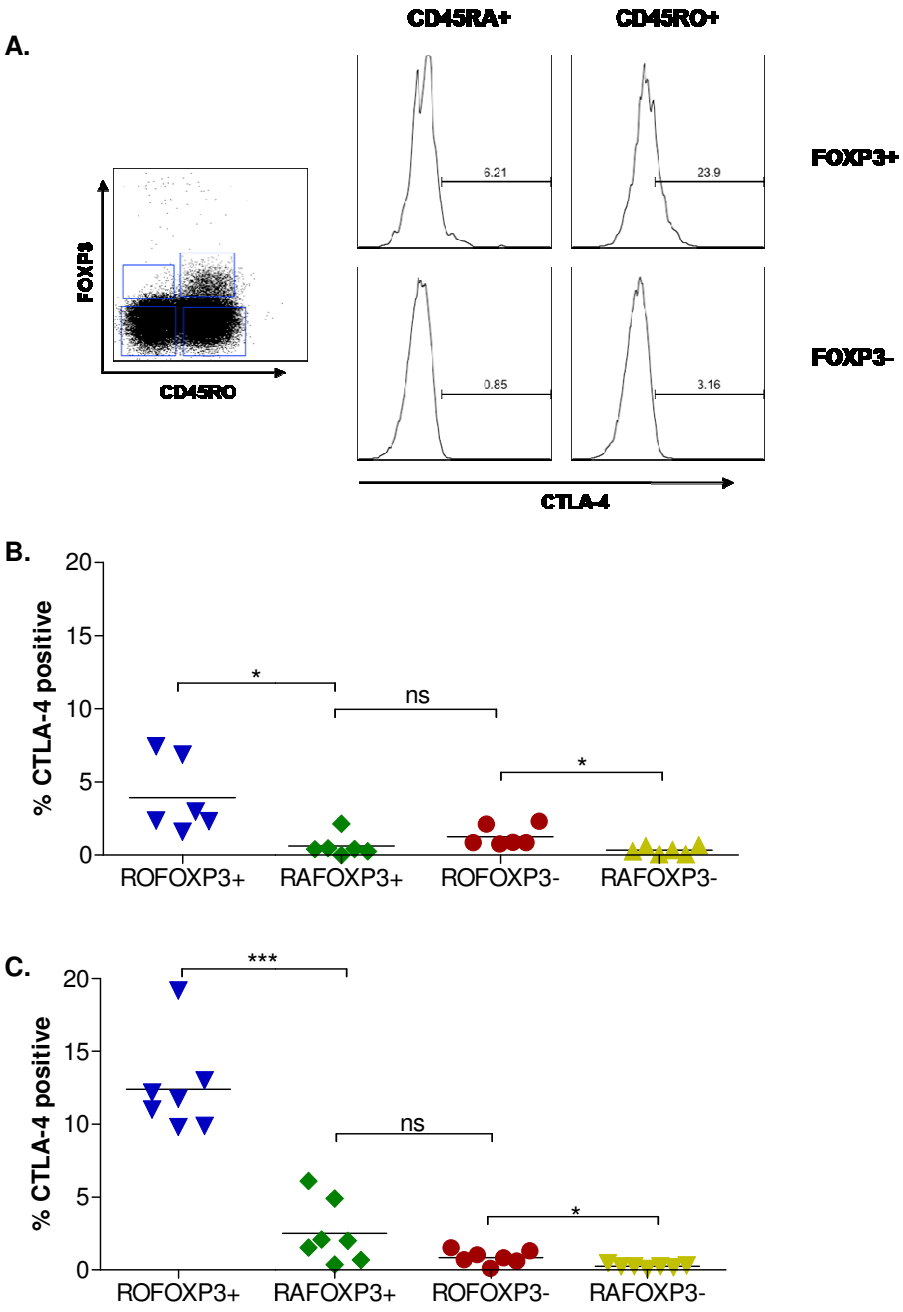


Figure 3.9. CTLA-4 expression by human CD4 Tregs and responder T cells.

PBMCs were stained *ex vivo* from healthy donors aged 22-50 years. **A.** Representative staining of CTLA-4 (intracellular) on FOXP3+ and FOXP3- CD4+ T cell subsets. **B.**Surface CTLA-4 expression by subsets of human CD4 T cells. CTLA-4 was detected by incubation of live cells with anti-CTLA-4 antibody for 30mins at 37℃ in the dark. This was followed by staining for other markers. (n=6) **C.** Intracellular CTLA-4 expression by subsets of human CD4 T cells. Anti-CTLA-4 antibody was added along with anti-FOXP3, after cell fixation and permeabilisation, to give intracellular as well as surface staining. (n=7)

3.4.3 Naïve and memory Tregs are equally capable of suppressing proliferation and cytokine production by responder CD4+ T cells

To investigate whether the difference in phenotype between memory and naïve Tregs has an effect on their capacity to suppress responders, we performed suppression assays using MACS-isolated CD45RA+ or CD45RO+ Tregs as suppressors. Figure 3.10 shows the purities of these cells. We investigated suppression of responder cell proliferation, using incorporation of ^{3}H-thymidine as a measure, as well as suppression of responder cells' production of interferon-gamma (IFN-γ) and IL-2. As shown in Figure 3.11, when used at a ratio of 1:1 with responders, CD45RA+ and CD45RO+ Tregs were equally capable of suppressing both proliferation (CD45RA+ mean 85.7%±3.9; CD45RO+ mean 83.2%±2.9; n=5; p=0.3978) and cytokine production (IFNγ: CD45RA+ mean 40.7%±16.5; CD45RO+ mean 30.2%±10.9; p=0.6298. IL-2: CD45RA+ mean 24.0%±12.4; CD45RO+ mean 13.7%±9.0; p=0.5462) despite their differing expression of both CTLA-4 and CD39. Nor did there appear to be any difference in the suppressive capacities of naïve and memory Tregs when used at lower, more physiological ratios in preliminary experiments (data not shown). This might result from different mechanisms of suppression, or if CTLA-4 and CD39, both activation markers, are upregulated upon activation of CD45RA+ Tregs, allowing them to suppress. We therefore investigated the kinetics of CD39 upregulation on stimulation of CD39- Tregs.

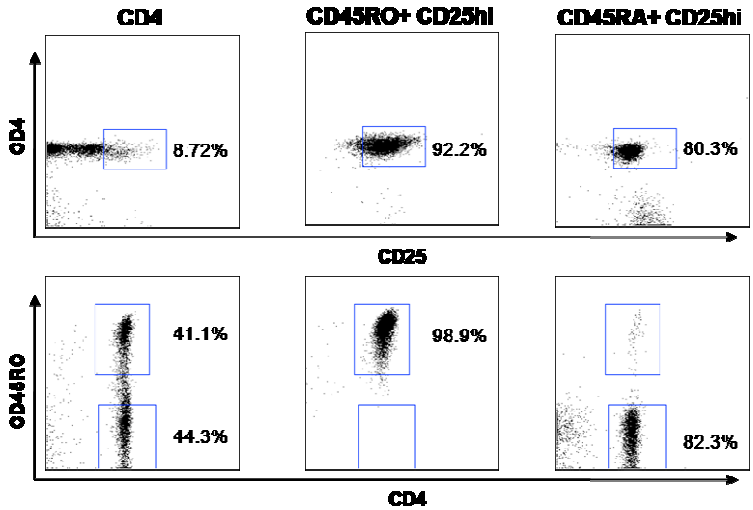


Figure 3.10. Purities of magnetically-isolated CD45RA+ and CD45RO+ CD25^{hi} Tregs

CD45RA+ and CD45RO+ cells were isolated from CD4+ cells using magnetic beads conjugated to anti-CD45RO or anti-CD45RA, respectively. The mixture was run through a magnetic column and the required cells passed through, then incubated with anti-CD25 antibody-conjugated magnetic beads to obtain CD25^{hi} cells.

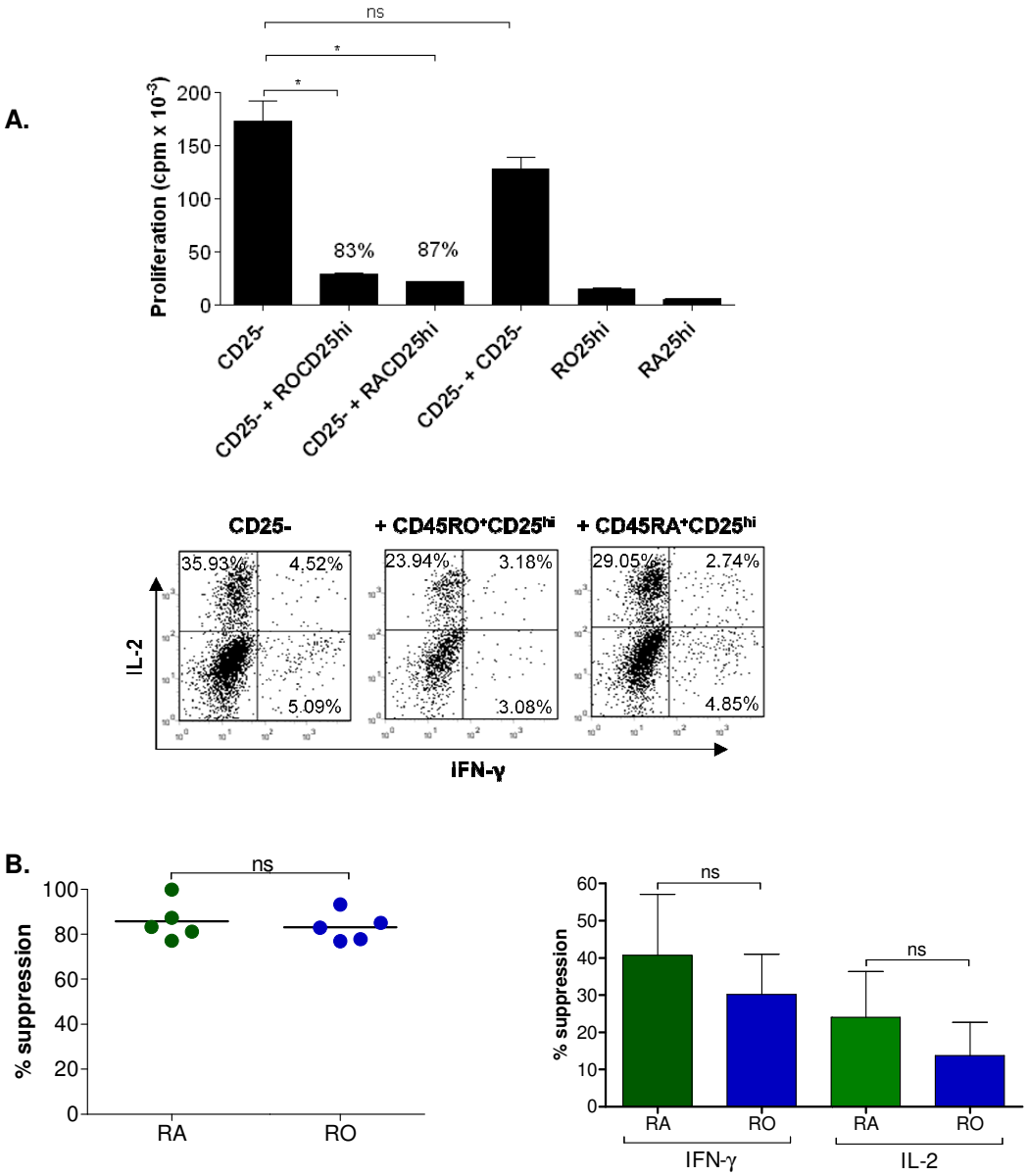


Figure 3.11. CD45RA+ and CD45RO+ Tregs are equally suppressive at a ratio of 1:1 with target cells.

CD45RA+ and CD45RO+ CD25^{hi} CD4+ T cells, as well as CD25- responder T cells, were isolated using MACS. Proliferation of responders, and production of IFN-γ and IL-2 by them, was measured when cultured alone and at a ratio of 1:1 with each subset of Tregs. Incorporation of ^{3}H-thymidine was used as a measure of proliferation. Cytokine production was measured by stimulating cells with anti-CD3 and anti-CD28 for two days, then restimulating with PMA and ionomycin for 3h while blocking export with brefeldin-A, followed by intracellular staining. **A.** *Top:* representative data from a suppression assay measuring proliferation, showing responder T cells alone, cocultured with CD45RO+ and CD45RA+ Tregs, cultured with more responders, and CD45RA+ and CD45RO+ Tregs cultured alone. Cells were stimulated with anti-CD3, anti-CD28 beads and cultured at 50,000 cells/well. Each condition was set up in triplicate. *Bottom:* representative dotplots showing IFN-γ and IL-2 production by CD4+ responder T cells in the absence or presence of CD45RA+ or CD45RO+ Tregs. Responders were CFSE-stained to distinguish them from Tregs. **B.** Cumulative data: *left,* suppression of responders' proliferation (n=5); *right*, suppression of cytokine production (n=5).

3.4.4 CD39 is not required for suppression by human FOXP3+ Tregs

CD39 has previously been shown to be necessary for *in vitro* suppression by murine FOXP3+ regulatory T cells [215, 216]. To determine whether it is required for the function of their human counterparts, CD39+ cells were depleted from the Treg population by MACS (Figure 3.12A). The CD39- Tregs' capacity to suppress proliferation and IFN-γ and IL-2 production was compared to total Tregs. No difference was found in the suppressive capacity of total and CD39- Tregs (Figure 3.12B).

CD39 is an activation marker, and cells that were initially CD39- could therefore upregulate it upon stimulation. Consequently, cells were stained for CD39 expression again at the time the readout of suppression was taken; no upregulation of CD39 was observed on the CD39- Tregs by this point, day 2 (Figure 3.12C), suggesting that CD39 is not a necessary mediator of human FOXP3+ Treg suppression.

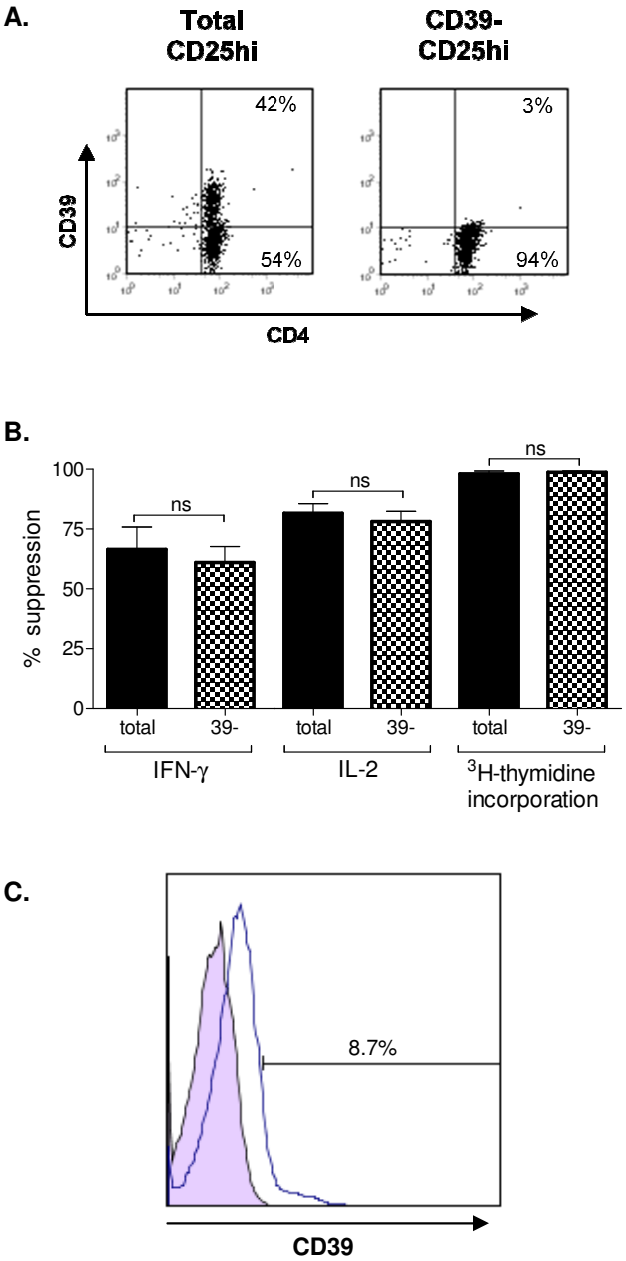


Figure 3.12. CD39 expression is not necessary for suppression by human CD25^{hi} regulatory T cells.

CD39-expressing cells were depleted from the Treg population and their suppressive capacity compared to that of total Tregs from the same donor. **A.** Representative dotplots showing CD39 expression by Tregs before and after CD39-depletion via MACS. **B.** Cumulative data showing suppression of IFN-γ and IL-2 production and proliferation by CD4+CD25- responder T cells by total (black bars) and CD39- (checked bars) Tregs. Results are cumulative from three independent experiments. **C.** Representative histogram showing CD39 expression by CD39- Treg population before stimulation (lilac-filled) and after incubation with beads and responders for two days (dark blue outline).

3.5 Induction of FOXP3+ Tregs via anergy

Earlier data in this study indicate that CD45RA+ Tregs proliferate to a greater degree in the old, and hence contribute to maintenance of the Treg pool. However, by the age of 80 the size of this subset is extremely small, limiting its potential to replenish the whole pool of Tregs alone, even with the increased proliferation we observed. It is known that Tregs can be induced peripherally via several mechanisms, including TGF-β and retinoic acid [78, 211, 232, 235]. However, work by our group has suggested that anergised responder CD4+ T cells may also have suppressive capacity [314].

In order to explore the potential of anergy induction to generate regulatory T cells, we anergised PPD-specific CD4 T cells (see Materials and Methods) using various concentrations of plate-bound anti-CD3 without any costimulation. Figure 3.13A shows the impaired response of these anergised cells to restimulation with PPD. We then analysed the PPD-stimulated proliferation of non-anergised PPD-specific CD4+ T cells, with and without the addition of anergised cells. As seen in Figure 3.13B, proliferation of the unanergised responders was reduced by the anergised cells, particularly those anergised with higher concentrations of anti-CD3. Figure 3.14 shows an increase in expression of FOXP3 on the protein level in these cells; this acquisition of FOXP3 expression appears to be associated with acquisition of suppressive capacity, suggesting that these are induced regulatory T cells.

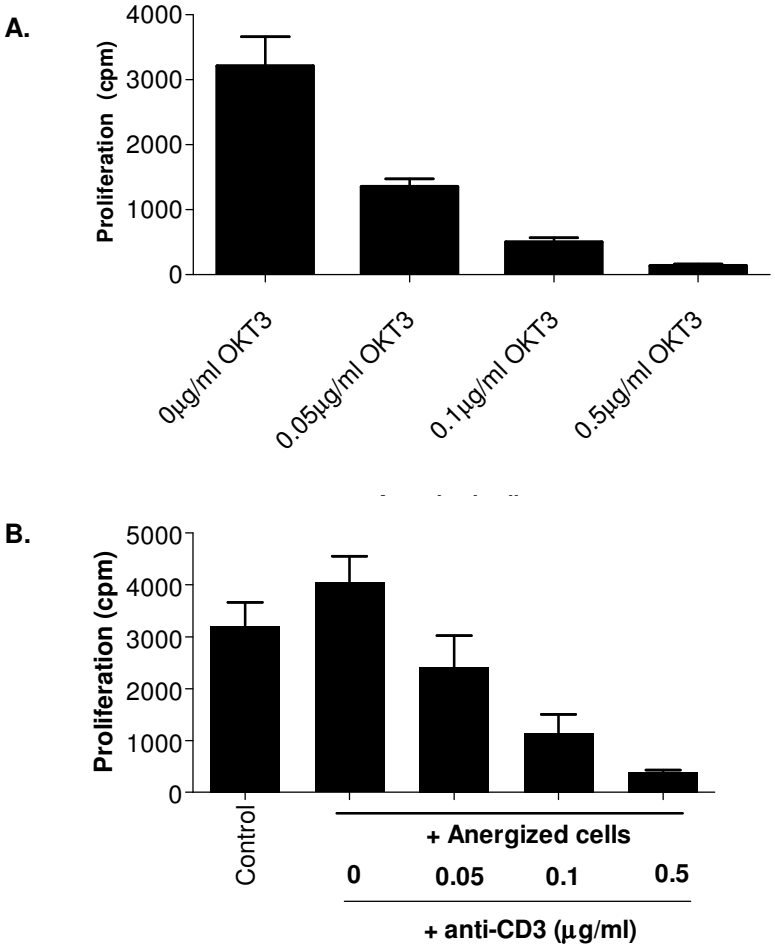


Figure 3.13. Anergised CD4+ T cells acquire suppressive ability.

PPD-specific CD4+ T cells were anergised with varying concentrations of anti-CD3 (OKT3), then cocultured with APCs and 1µg/ml PPD for 5 days before proliferation was analysed. **A.** Proliferation (measured by incorporation of ^{3}H-thymidine) of T cells anergised with 0, 0.05, 0.1 and 0.5 µg/ml anti-CD3. **B.** Anergised cells were cultured with non-anergised PPD-specific CD4+ T cells, along with 1µg/ml PPD and APCs. Graph shows proliferation of non-anergised PPD specific cells alone (Control), and then proliferation on coculture of these cells with T cells anergised with increasing concentrations of anti-CD3.

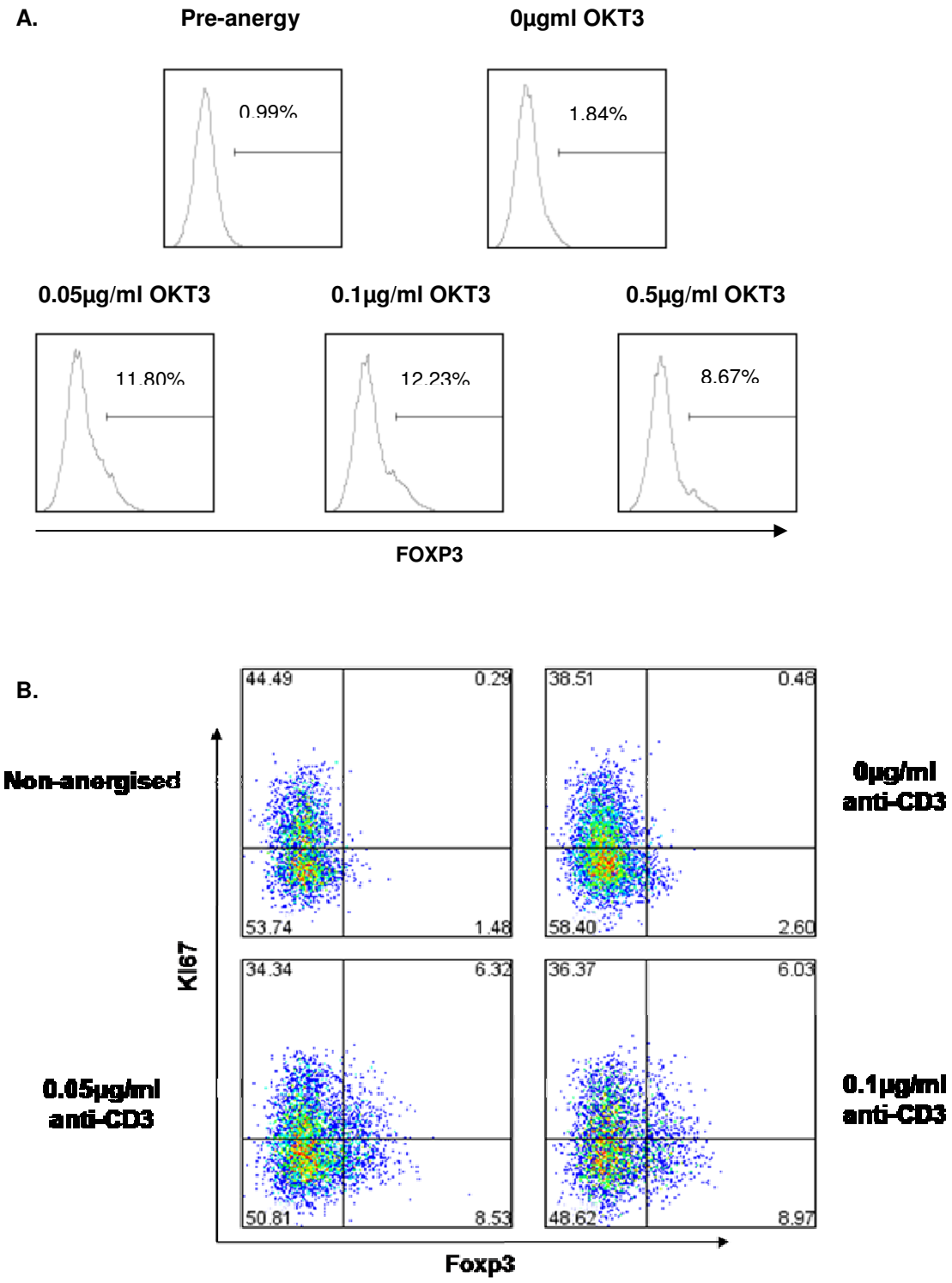


Figure 3.14. Anergised cells acquire expression of FOXP3.

Anergised cells were tested for protein level expression of FOXP3 and the proliferation marker, Ki67. **A.** Histograms showing expression of FOXP3 before and after anergising the cells using varying concentrations of anti-CD3. **B.** Dotplots showing expression of FOXP3 and Ki67 on cells pre- and post-anergy. Top left: non-anergised cells. Top right: cells incubated with 0µg/ml anti-CD3. Bottom left: cells incubated with 0.05µg/ml anti-CD3. Bottom right: cells incubated with 0.1µg/ml anti-CD3.

3.6 Discussion

This chapter concerns the maintenance throughout life of the pool of regulatory cells within the CD4+ T cell subset. These cells, known as Tregs, are thought to be generated within the thymus; however, in adults, during and after thymic involution, it is postulated, although still currently controversial, that peripheral conversion of conventional CD4+ T cells may play a role in maintaining the Treg pool [228, 340]. The precise contributions of the thymus and periphery in maintaining the Treg population throughout life are a still a matter of debate.

In the first part of the chapter we sought to characterise the composition of the Treg pool at different stages of adult life. At around age 20, a significant proportion of Tregs was found to express CD45RA and can hence be considered to be naïve; however, by the age of 80, this has declined to almost zero. Recent thymic emigrants (RTEs), CD45RA+ CD31+ cells, are postulated to be truly naïve, whereas cells which express CD45RA but not CD31 are thought to have divided [69, 70]. An even smaller proportion of the Treg pool was found to have an RTE phenotype and this proportion also declined with age. However, the proportion of the CD4+ T cell pool as a whole that has a Treg phenotype increases slightly with age, despite maintenance of CD4+ T cell numbers in the peripheral blood with age (data not shown) suggesting that compensatory mechanisms are keeping the Treg pool replenished despite the declining capacity of the thymus. This could be peripheral conversion of conventional CD45RO+ CD4+ T cells to CD45RO+ Tregs, or proliferation of thymically-produced Tregs with concomitant conversion to express CD45RO.

Previous work using deuterated glucose has demonstrated a high turnover rate among CD45RO+ Tregs, with an estimated doubling time of 8 days (compared to 24 days among conventional CD4+ T cells) [228]. In order to further elucidate the proliferation rate of Tregs, and to investigate proliferation of the naïve subset, samples were stained for the nuclear marker of proliferation, Ki67. This was found in a very high proportion of CD45RO+ Tregs: a mean of 18% were Ki67-positive. Proliferation was significantly lower among CD45RA+ Tregs, although still higher than that observed among naïve responders, with 5% actively dividing *ex vivo.* Naïve Tregs have been shown to have long telomeres [221], in contrast to their memory counterparts [228], so this higher rate of proliferation in CD45RA+ Tregs than responders, and their consequent upregulation of CD45RO as observed after stimulation *in vitro*, is highly likely to be a contributor to the maintenance of a Treg presence in the old, particularly given the higher rate of

proliferation observed among RTE Tregs in older subjects. The higher levels of Ki67 seen among RTE Tregs than CD31- Tregs (and among RTE responders compared to CD31- naive responders) may also be caused by residual expression of Ki67 after thymic proliferation [341].

A correlation was seen between smaller naïve Treg pool size and increased proliferation of the naïve cells within it, which confirms earlier studies [339]. This finding, that smaller pools of naïve T cells tend to proliferate more, may also go some way towards explaining the high rate of proliferation among CD45RA+ Tregs in general, compared to CD45RA+ or CD45RO+ responder T cells. Even in young adults, the CD45RA+ Treg pool is extremely small. An interesting avenue of investigation might be the proliferation rates among CD45RA+ Tregs in cord blood, where the population is much larger. The high rate of proliferation among CD45RO+ Tregs, however, cannot be explained by the small size of the population: even in young adults, this population is much larger than the CD45RA+ pool and yet displays much higher rates of proliferation. It is clear that these cells have a propensity to divide frequently, although it must be stressed that our data cannot rule out the existence of long-lived and slowly-dividing memory Tregs, which may also help to sustain the Treg population. Mathematical models may help to explain the processes taking place.

The proportion of the Treg pool in adults generated directly in the thymus as opposed to peripherally by conversion is still a major subject of investigation. In order to better describe the naïve and memory Treg subsets in younger adults, we investigated their expression of certain Treg markers, as well as their functional capacity. A marked difference in the expression by the two subsets of both CD39 and CTLA-4 was observed, despite their suppressive equivalence at a 1:1 ratio with their targets. More physiological ratios were only investigated in a preliminary fashion (data not shown), although these did not show significant differences in suppression. Additionally, the *in vitro* suppression assay was performed in an APC-free system. A more physiological set-up may yet identify differences in suppressive capacity and further work is needed to investigate the efficiency with which CD45RA+ and CD45RO+ Tregs suppress when they are much lower in number than their targets.

Although CD39 is postulated in this study to be unnecessary for human Treg suppression in general, there nevertheless remains the possibility that CD45RA+ and CD45RO+ Tregs – especially if the latter are converted from peripheral responder T cells – may function via different mechanisms: the importance of CTLA-4 to Treg

function is strongly supported by numerous studies in both mouse and man. It is important, however, to note that CTLA-4 is an activation marker, and may be upregulated on CD45RA+ Tregs prior to suppression. Time did not permit the kinetics of CTLA-4 upregulation to be investigated in this study, but this experiment may be informative to future work. In addition, the study on the importance of CD39 molecule to human Treg suppression used CD39- isolated via MACS. These may have been from a different source to the CD39+ cells: they may have been largely naïve, and hence potentially suppressing via a different mechanism. Knocking down the expression of CD39 in cells that previously expressed the marker would be a more reliable method of determining the importance of CD39 to the function of all human Tregs: siRNA studies would be a good way of doing this, as well as blocking anti-CD39 antibody.

A number of studies have demonstrated that suppressive function and FOXP3 expression can be induced in conventional T cells as a result of stimulation in the presence of TGF-β or retinoic acid or both. This has been demonstrated both *in vitro* and, in mice, *in vivo*. In this chapter we investigated the induction of anergy as a potential conversion mechanism and demonstrated that it can indeed generate suppressive capacity as well as protein expression of the Treg marker, FOXP3. A further avenue of investigation would be the potential involvement of T:T presentation in the induction of regulatory T cells *in vivo*. T:T presentation occurs as a consequence of upregulation of MHC Class II on activated human T cells [203], although this does not occur in mice. Expression of MHC Class II enables these T cells to present antigenic peptides to other CD4 T cells. This phenomenon has been shown to result in anergy induction in the cells receiving the signal [170, 171], although some studies have reported successful activation [172] and the mechanism and role of T:T presentation are not yet entirely clear. An immune response generates large numbers of T cells in close proximity in the affected tissue. It is possible that T:T presentation could induce anergy, and therefore T regulatory capacity, in a number of CD4 T cells present at the site as a way of dampening the immune response once it has peaked. This mechanism also provides a potential source of additional Tregs, which could be antigen-specific, generated via anergy induction during the course of normal immune responses during life and replenishing the Treg pool.

Overall, it seems that the thymus, although very small in productive size at advanced ages, is still able to contribute somewhat to the Treg pool in old age. However, the conversion – perhaps by previously-postulated mechanisms or by anergy induction – of peripheral memory responder T cells is still likely to be important. This is supported by

gene expression analyses, which suggest a strong lineage relationship between CD45RO+ Tregs and CD45RO+ responder T cells. Conversion of peripheral CD4+CD25- T cells to a regulatory phenotype could continuously support Treg numbers and potentially provide antigen-specific Tregs in response to encounters with harmless antigens. Indeed, the phenotypic differences between CD45RA+ and CD45RO+ Tregs appear to be quite striking, despite their functional equivalence at a 1:1 ratio. Further work is required to fully evaluate the sources and suppressive mechanisms of these cells.

Chapter 4. Migration of human FOXP3+ Tregs

4.1 Introduction

Tregs, like all T cells, circulate in the blood and lymph but must leave these vessels in order to exert their effects in the tissues. They must therefore migrate through the endothelium lining these vessels. Transendothelial migration is a multistep process initiated when surface molecules on the lymphocyte make contact with selectins on the surface of the endothelium. This slows the T cell down, a process termed 'rolling' [106, 113]. The endothelium expresses E-selectin and P-selectin in response to inflammatory stimuli [109], and these bind glycoproteins on the T cell such as CLA (bound by E-selectin) [120, 121]. Firm adhesion of the T cell to the endothelial lining is then possible provided the T cell expresses the correct receptors for the integrins present on endothelium in that particular tissue, since integrin expression is tissue-specific.

After adherence, the T cell can move through the endothelium in a process termed diapedesis [107] and can then travel through the tissue along chemokine gradients [138]. This complex process means that in order to reach a particular tissue, a T cell must express all the correct receptors to attach to the endothelium and for the appropriate chemokines.

We first investigated the migration of human Treg subsets by looking at their expression of various chemokine and other homing receptors, to establish where they were most likely to migrate to; we then studied the ability of Treg and T-responder subsets to migrate through dermal endothelium *in vitro*, and finally we investigated the *in vivo* localisation of these cells in two different tissues: the skin, an accessible organ frequently prone to inflammation, and the bone marrow, a primary lymphoid organ.

4.2 Chemokine receptor expression

The chemokine receptor expression of CD45RO+ and CD45RA+ Tregs was investigated. As shown in Figure 4.1, we found high levels of CCR7 expression on both subsets of Tregs; this chemokine receptor binds CCL19 and CCL21, produced by cells in lymphoid organs [139]. T cells expressing high levels of CCR7 therefore have lymph node homing characteristics.

CLA and CCR4, in contrast, are skin-homing markers [121, 138, 142]. CLA is a surface marker that binds E-selectin and is expressed by over 90% of skin-infiltrating lymphocytes [121]. CCR4 binds TARC (also known as CCL17), which is released by dermal keratinocytes [142]. As seen in Figures 4.2 and 4.3, both receptors were expressed by a large proportion of CD45RO+ Tregs (mean 42.72%±4.7 positive for CLA; 74.87%±5.1 positive for CCR4), but showed significantly lower expression on the CD45RA+ subset ($p<0.0001$ for CLA, $p=0.0003$ for CCR4). However, expression of CLA by CD45RA+ Tregs was significantly higher than that by CD45RA+ conventional T cells, and expression of CCR4 tended to be higher on the regulatory cells (CLA mean 16.4%±4.7 for CD45RA+FOXP3+ cells, mean 2.029%±0.6 for CD45RA+FOXP3- cells, $p=0.0061$; CCR4 mean 20.97%±8.3 CD45RA+FOXP3+ cells positive, mean 3.53%±1.4 CD45RA+FOXP3- cells positive; $p=0.068$). This suggests that CD45RO+ Tregs are more likely to home to the skin than CD45RA+ Tregs.

We also investigated expression of the bone marrow homing chemokine receptor, CXCR4. This protein binds to the chemokine CXCL12 or SDF-1, produced in large quantities in the bone marrow [141, 342]. We found higher expression of CXCR4 on both CD45RA+ Tregs and CD45RA+ responders compared to CD45RO+ cells (Figure 4.4), suggesting that naïve T cells have a strong propensity to home to the bone marrow. (MFI of CXCR4 on CD45RA+FOXP3+: 53.96±12.4; CD45RO+FOXP3+: 15.99±2.6; $p=0.009$. MFI of CXCR4 on CD45RA+FOXP3-: 26.66±3.0; CD45RO+FOXP3-: 14.45±2.3; $p=0.0005$.) CXCR4 expression on CD45RA+FOXP3+ cells was also significantly higher than on CD45RA+FOXP3- cells ($p=0.05$).

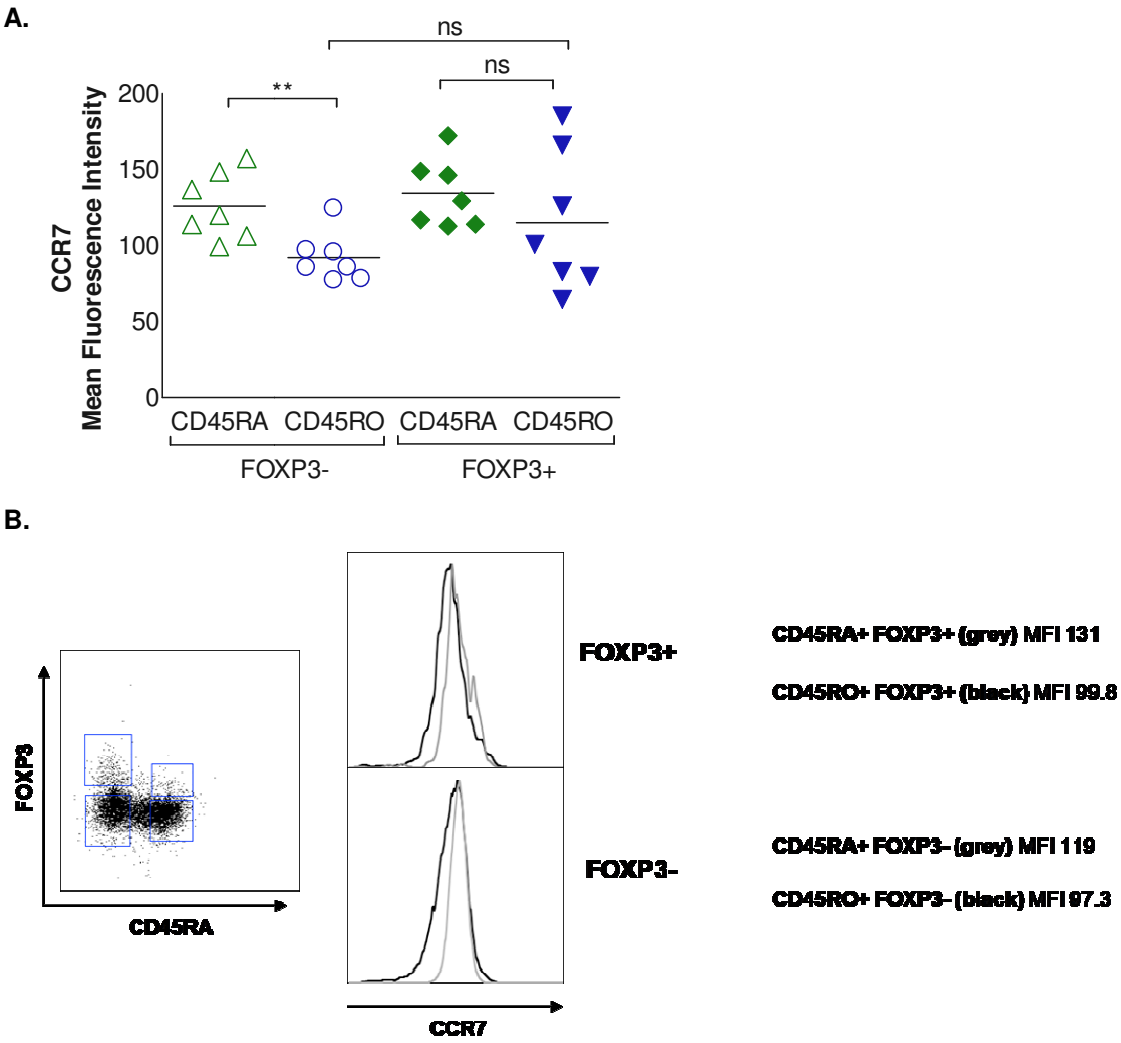


Figure 4.1. CCR7 expression by subsets of responder and regulatory T cells.

A. Mean fluorescence intensity of CCR7 expression on CD45RA+ and CD45RO+ responder (FOXP3-) and regulatory (FOXP3+) CD4+ T cells (n=7). **B.** Representative staining of CCR7 by naive and memory subsets of FOXP3+ and FOXP3- CD4+ cells.

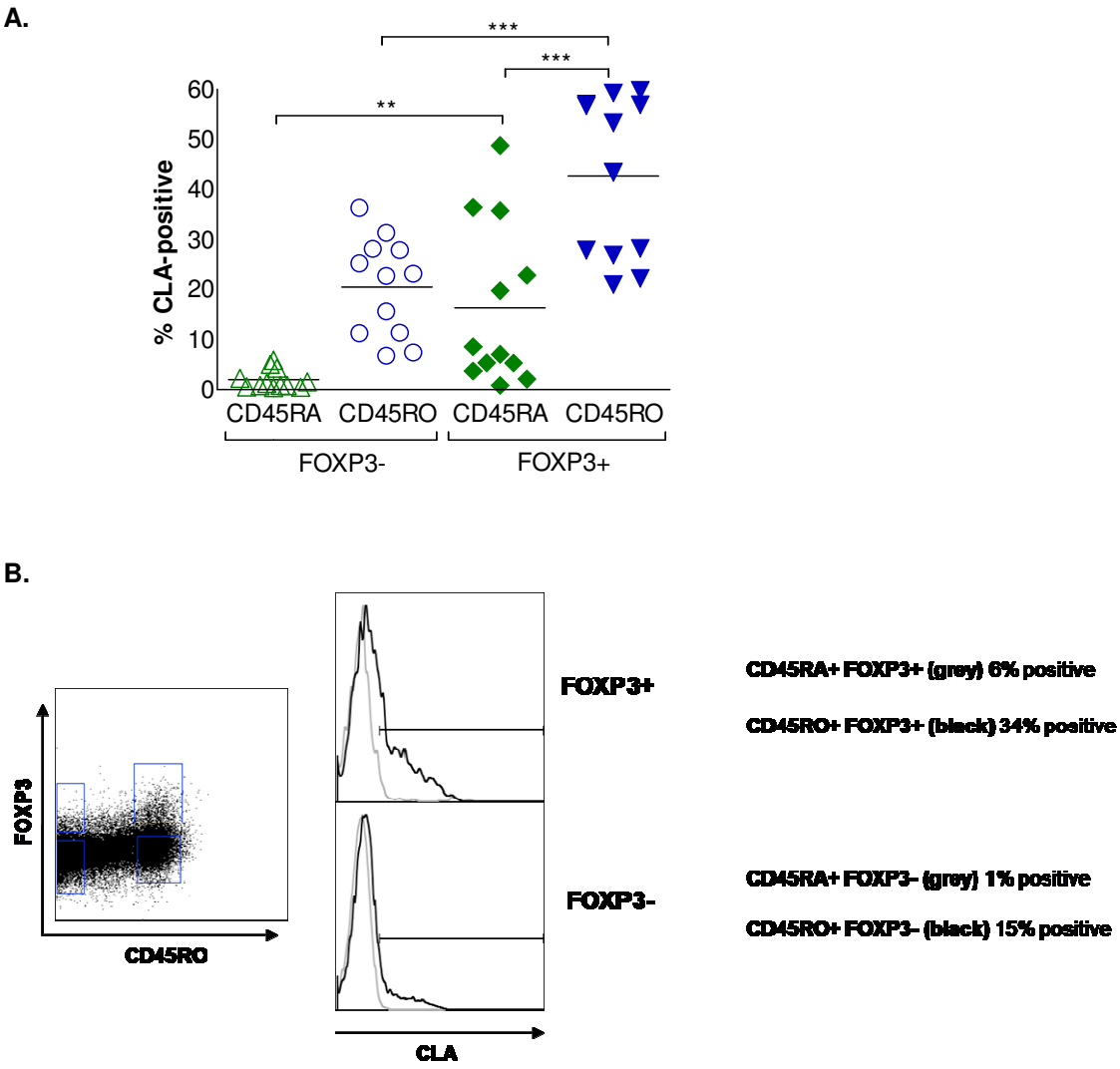
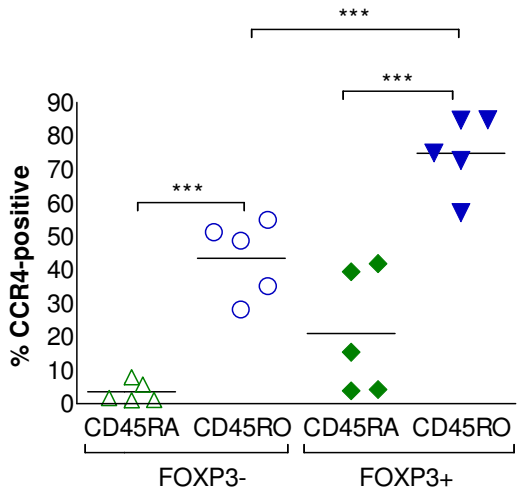


Figure 4.2. CLA expression by subsets of responder and regulatory T cells.

A. Mean fluorescence intensity of CLA expression on CD45RA+ and CD45RO+ responder (FOXP3-) and regulatory (FOXP3+) CD4+ T cells (n=7). **B.** Representative staining of CLA by naive and memory subsets of FOXP3+ and FOXP3- CD4+ cells.

A.



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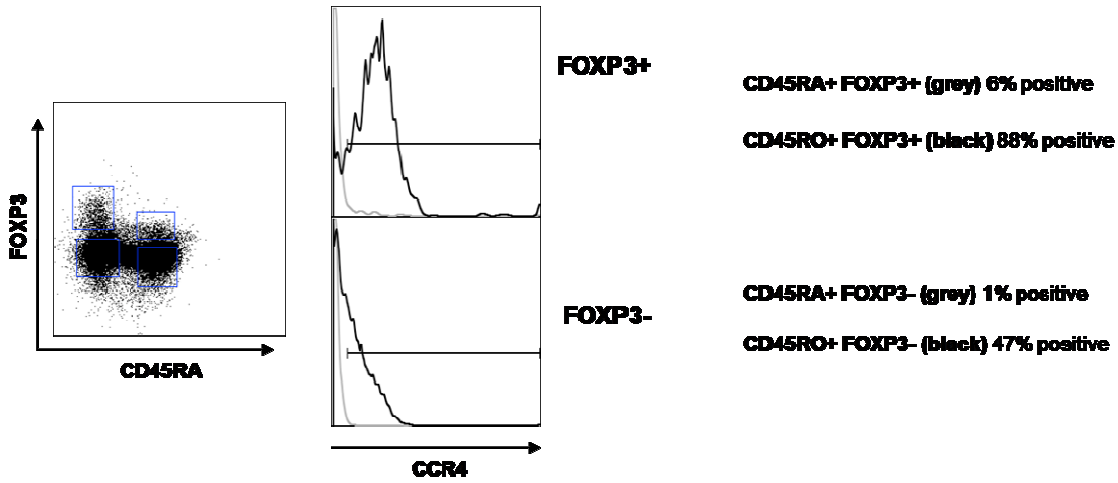


Figure 4.3. CCR4 expression by subsets of responder and regulatory T cells.

A. Mean fluorescence intensity of CCR4 expression on CD45RA+ and CD45RO+ responder (FOXP3-) and regulatory (FOXP3+) CD4+ T cells (n=7). **B.** Representative staining of CCR4 by naive and memory subsets of FOXP3+ and FOXP3- CD4+ cells.

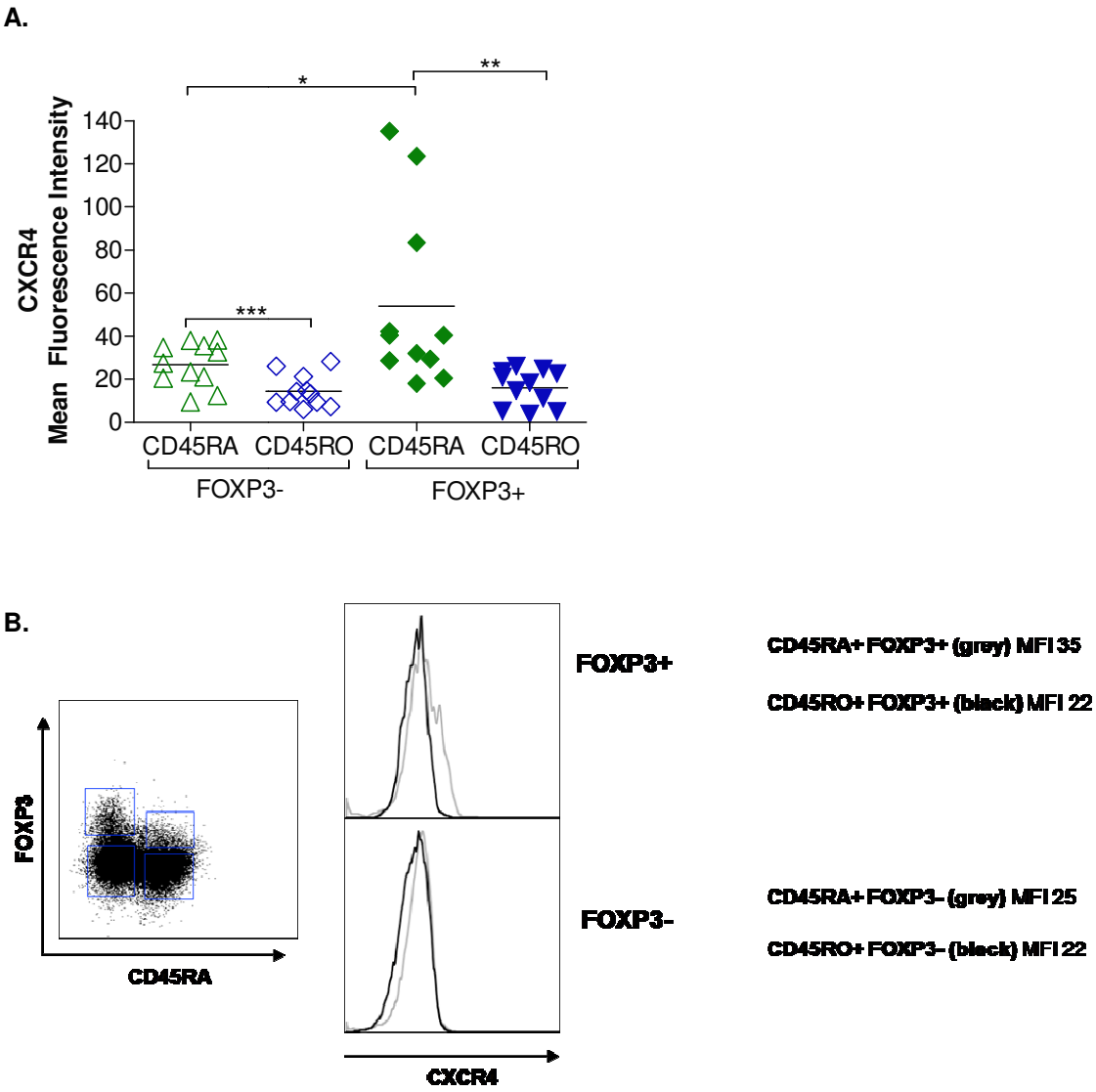


Figure 4.4. CXCR4 expression by subsets of responder and regulatory T cells.

A. Mean fluorescence intensity of CXCR4 expression on CD45RA+ and CD45RO+ responder (FOXP3-) and regulatory (FOXP3+) CD4+ T cells (n=7). **B.** Representative staining of CXCR4 by naive and memory subsets of FOXP3+ and FOXP3- CD4+ cells.

4.3 Transendothelial migration of Tregs *in vitro*

A greater proportion of CD4+ Tregs than responders expresses the skin-homing markers CLA and CCR4, implying that regulatory T cells should have a greater capacity than responders to migrate into the skin across dermal endothelium. To test this hypothesis, we followed their migration using an *in vitro* system using primary endothelial cells. We first compared the migration of CD45RO+ responder CD4+ T cells and CD45RO+ Tregs over monolayers of stimulated and unstimulated endothelium. We then extended our investigations to include the CD45RA+ subsets of both responder T cells and Tregs.

The cells were added to a stimulated (with IFN-γ and TNF) or unstimulated dermal endothelial monolayer and the extent of migration after 2 hours and 4 hours was estimated using time-lapse microscopy. Figure 4.5 shows a number of still images from time-lapse videos illustrating the extent of migration seen by Tregs or responders on stimulated and unstimulated endothelium (also see Movies 1-4 on the attached CD or at www.mediafire.com/migrationvideos). Migrated cells appear darker, wider and flatter than cells that are still on the upper surface of the endothelium, with no 'halo'. Overall, no difference was seen in the migratory capacity of CD45RO+ Tregs and responder T cells to cross either stimulated or unstimulated dermal endothelium (Figure 4.6A), after 2 hours (mean percentage migrated over stimulated endothelium: CD45RO+FOXP3-: 22.63%±6.3; CD45RO+FOXP3+: 28.57%±5.4; p=0.6304; over unstimulated endothelium: CD45RO+FOXP3-: 4.833%±2.1; CD45RO+FOXP3+: 2.067%±0.72; p=0.3408) or 4 hours (mean percentage migrated over stimulated endothelium: CD45RO+FOXP3-: 20.46%±4.7; CD45RO+FOXP3+: 13.74%±3.5; p=0.2760; over unstimulated endothelium: CD45RO+FOXP3-: 1.70%±1.7; CD45RO+FOXP3+: 4.0%±0.51; p=0.3848).

On investigation of the transendothelial migratory capacity of CD45RA+ and CD45RO+ cells among responders and regulatory T cells, we found that naïve responders and regulatory T cells tended to migrate less efficiently than their CD45RO+ counterparts (Figure 4.6B and see Movies 5 and 6 on the attached CD), although due to a large degree of inter-patient variation, this trend was not significant (mean percentage migrated: CD45RO+FOXP3+ 14.13%±5.0; CD45RA+FOXP3+ 3.68%±2.6; p=0.08). CD45RA+ Tregs also migrated more efficiently than their CD45RA+ responder counterparts (mean percentage migrated: CD45RO+FOXP3- 15.85%±2.2; CD45RA+FOXP3- 0.638%±0.36; p=0.007).

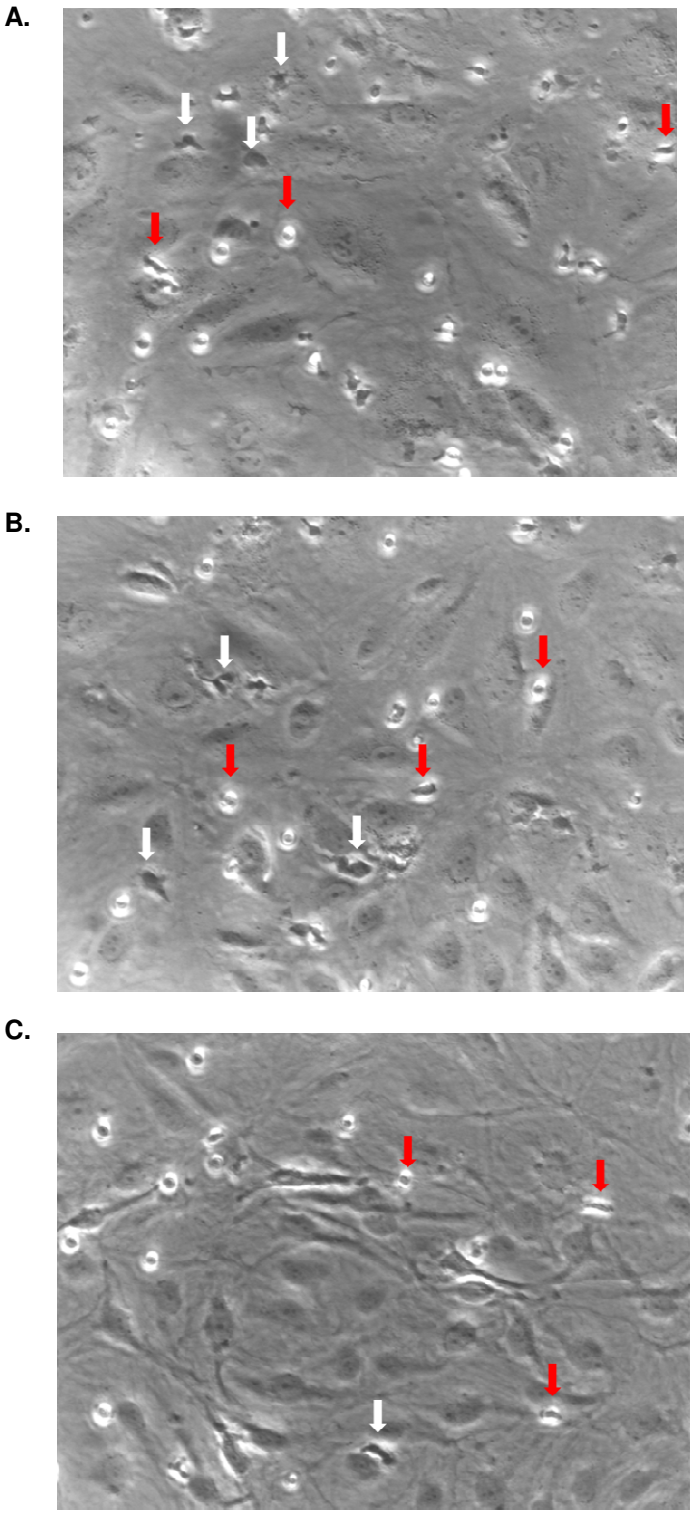


Figure 4.5. Transendothelial migration of human primary T cells.

Stills from time-lapse videos illustrating the appearance of migrated (indicated by white arrows) and unmigrated (indicated by red arrows) cells. Cells were loaded onto a primary endothelial cell monolayer and imaged after 2 hours. **A.** CD45RO+ CD4+ CD25- responders on stimulated endothelium. **B.** CD45RO+CD4+ $CD25^{hi}$ Tregs on stimulated endothelium. **C.** Responder CD4+ T cells on unstimulated endothelium.

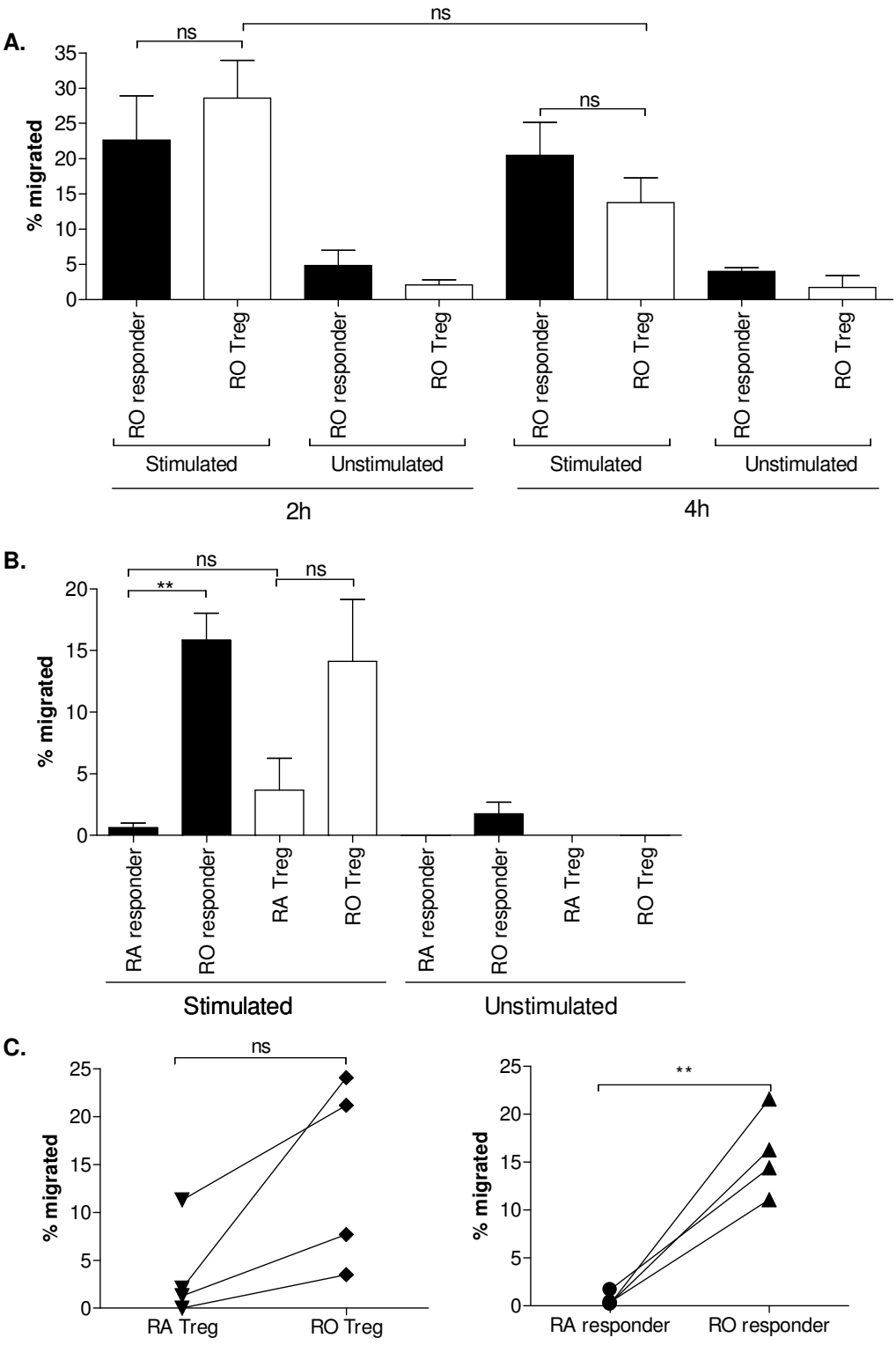


Figure 4.6. Comparison of transendothelial migration by subsets of responder and regulatory T cells.

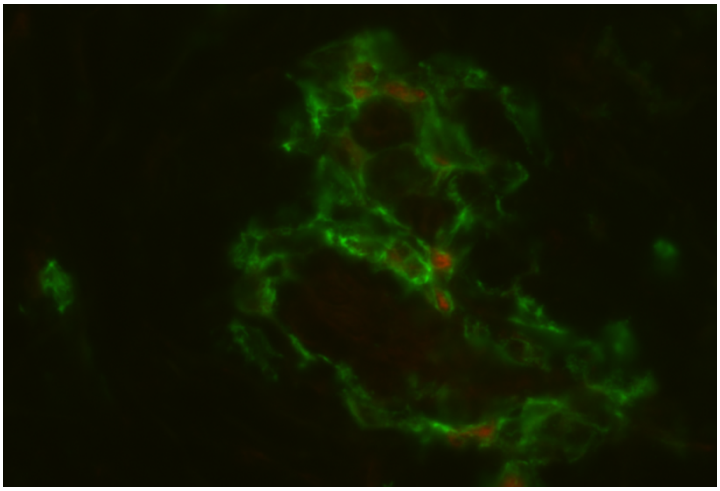
A. Migration of responder (CD25-) and regulatory ($CD25^{hi}$) CD4 T cells over stimulated and unstimulated primary endothelium at early (2 hours) and late (4 hours) timepoints. CD45RO+ cells were isolated to ensure a fair comparison. (n=3) **B.** Transendothelial migration by naïve and memory subsets of responder and regulatory CD4 T cells, over stimulated endothelium, after 4 hours. (n=4) **C.** Paired comparisons of migration by Treg (left) and responder (right) CD45RA+ and CD45RO+ subsets. (n=4)

4.4 Tregs in normal skin

Having established that CD45RO+ Tregs have a capacity to migrate through stimulated but not unstimulated dermal endothelium, we investigated tissue sections from normal, uninflamed skin to establish the extent of dermal infiltration by different subsets of Tregs *in vivo* under non-inflammatory conditions. Although low numbers of CD4+ T cells were found in unstimulated skin, a high proportion of these cells expressed FOXP3 (not shown).

We investigated normal skin sections using immunofluorescence microscopy to determine the proportions of FOXP3+ cells in normal skin expressing CD45RA and CD45RO; representative staining is seen in Figure 4.7A and B. In all infiltrates counted in sections from five different donors, only two FOXP3+ cells expressing CD45RA were detected. Table 6.1 shows the absolute counts obtained in sections from donors; young donors were used to look for CD45RA+FOXP3+ cells, but unfortunately had few FOXP3+ cells in many of the sections examined. There is however a clear increase in the propensity of skin FOXP3+ cells to be CD45RO+ compared to CD45RA+. This supports our previous data on the relative migration of CD45RA+ compared to CD45RO+ Tregs across endothelium *in vitro*.

A.



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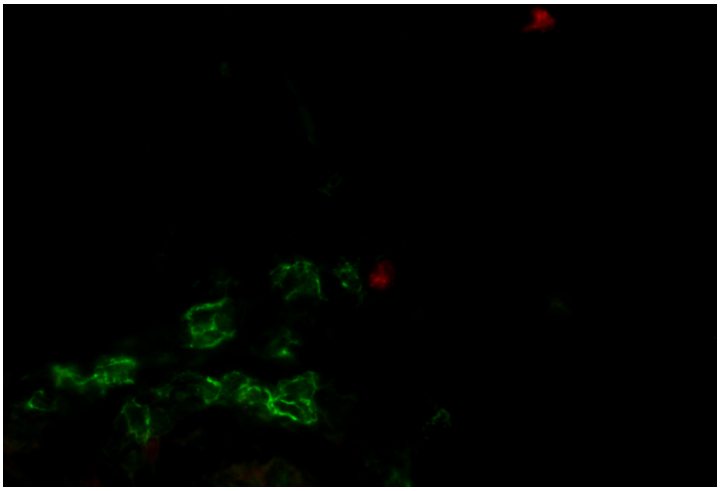
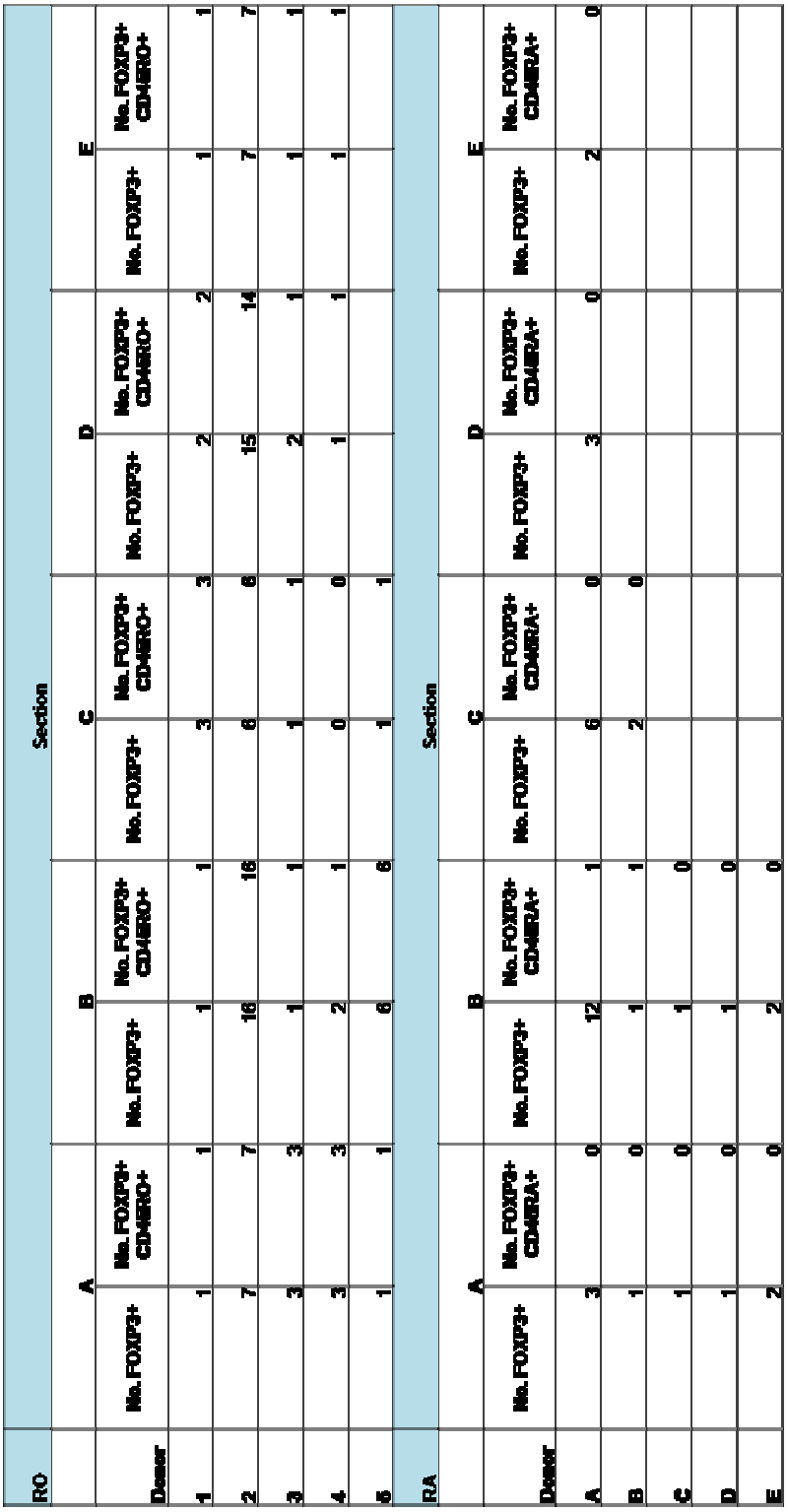


Figure 4.7. FOXP3+ cells are predominantly CD45RO+.

Punch biopsies were taken from healthy donors at sites of unchallenged skin, and sections stained for expression of FOXP3, CD45RA and CD45RO. **A.** Representative photograph showing expression of CD45RO (green) and FOXP3 (red) in unchallenged skin. **B.** Representative photograph showing expression of CD45RA (green) and FOXP3 (red) in unchallenged skin.

Table 6.1. Counts of CD45RA+ and CD45RO+ FOXP3+ cells in unchallenged skin.



RO		Section											
		A		B		C		D		E			
	Donor	No. FOXP3+	No. FOXP3+ CD45RO+	No. FOXP3+	No. FOXP3+ CD45RO+	No. FOXP3+	No. FOXP3+ CD45RO+	No. FOXP3+	No. FOXP3+ CD45RO+	No. FOXP3+	No. FOXP3+ CD45RO+		
	1	1	1	1	1	3	3	2	2	1	1		
	2	7	7	16	16	6	6	15	14	7	7		
	3	3	3	1	1	1	1	2	1	1	1		
	4	3	3	2	1	0	0	1	1	1	1		
	5	1	1	6	6	1	1						

RA		Section											
		A		B		C		D		E			
	Donor	No. FOXP3+	No. FOXP3+ CD45RA+	No. FOXP3+	No. FOXP3+ CD45RA+	No. FOXP3+	No. FOXP3+ CD45RA+	No. FOXP3+	No. FOXP3+ CD45RA+	No. FOXP3+	No. FOXP3+ CD45RA+		
	A	3	0	12	1	6	0	3	0	2	0		
	B	1	0	1	1	2	0						
	C	1	0	1	0								
	D	1	0	1	0								
	E	2	0	2	0								

4.5 CD45RA+ Tregs preferentially home to the bone marrow

CD45RA+, naïve, T cells are known to home preferentially to secondary lymphoid organs via high expression levels of CCR7 [106, 138]. High expression of the bone-marrow-homing chemokine receptor, CXCR4, was observed on CD45RA+ responder and especially regulatory CD4 T cells. The study was extended to investigate whether there was any evidence of preferential homing to the bone marrow by these cells.

4.5.1 CD45RA+ cells form a higher proportion of the FOXP3+ pool in the bone marrow than in the peripheral blood

Bone marrow, obtained from healthy bone marrow donors, was stained for expression of CD4, FOXP3 and CD45RA, at the same time as peripheral blood from the same donors. As shown in Figure 4.8, an enrichment of CD45RA+ cells was found among CD4+FOXP3+ cells, compared to the proportion observed among peripheral blood Tregs from the same donor. A corresponding reduction in the proportion of CD45RO+ Tregs was seen. No significant change in the proportion of CD45RA+ or CD45RO+ FOXP3- cells was seen compared to peripheral blood, however (not shown), indicating that this is a Treg-specific phenomenon.

4.5.2 CD45RA+FOXP3+ cells are proliferating at the same rate in bone marrow as in the peripheral blood

We hypothesised that the increased proportion of CD45RA+ cells among the bone marrow Treg population could be generated by increased proliferation among these cells, with the cytokine milieu of the bone marrow preventing loss of CD45RA expression. To establish whether this was the case, bone marrow and their paired peripheral blood samples were stained for expression of the nuclear marker of proliferation, Ki67. Figure 4.8 shows that the degree of proliferation among CD45RA+FOXP3+ cells from bone marrow compared to peripheral blood was not significantly different (bone marrow median: 5.36%; peripheral blood median: 5.24%; p=1.0; n=6). Proliferation among both CD45RO+ and CD45RA+ CD4+ responder subsets was also the same as that in peripheral blood. However, there was reduced proliferation of CD45RO+ FOXP3+ Tregs in the bone marrow compared to the peripheral blood (bone marrow median: 8.29%; peripheral blood median: 11.48%; p=0.002; n=6), which could lead to a relative decrease in the proportion of CD45RO+ cells in the bone marrow Treg pool.

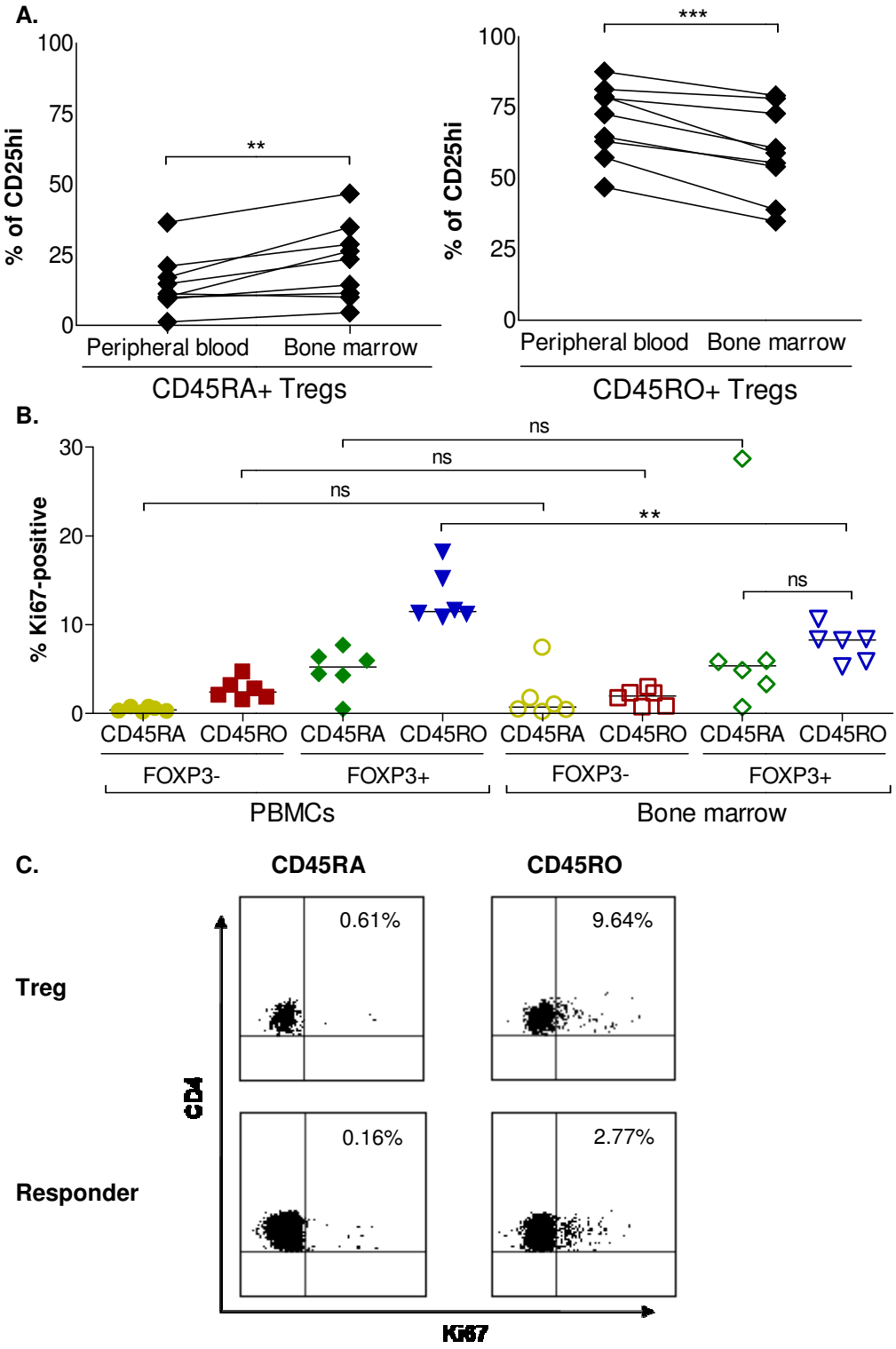


Figure 4.8. CD45RA+ Tregs preferentially home to the bone marrow.

Bone marrow and PBMC samples from the same healthy donors were investigated for the prevalence of Treg subsets and proliferation. **A.** Paired graphs showing the proportions of Tregs expressing CD45RA (left) and CD45RO (right) in bone marrow and peripheral blood. **B.** Graph showing Ki67 expression by Treg and responder CD45RA+ and CD45RO+ subsets in peripheral blood and bone marrow. **C.** Representative dotplots showing Ki67 expression by CD45RA+ and CD45RO+ Treg and responder subsets in bone marrow.

4.6 Discussion

Previous studies on human Tregs have focused on cells isolated directly *ex vivo* from peripheral blood, or induced *in vitro*, but much less is known about the presence and behaviour of these cells in the tissues. In this study we set out to investigate the migratory behaviour of these cells within the healthy individual. We found that CD45RO+ Tregs were more likely than the CD45RA+FOXP3+ subset to home to the skin, with a very large proportion expressing CLA and CCR4. Intriguingly, however, CD45RA+ Tregs do express higher levels of both markers than CD45RA+ responders. This led us to investigate whether CD45RA+ FOXP3+ cells were present in healthy, uninflamed skin.

The migration of CD45RO+ Tregs and CD45RO+ responders through a monolayer of dermal endothelium was observed *in vitro*. We isolated CD45RO+ cells from both subsets for a fair comparison. We found that Tregs and responders had an equal migratory capacity, despite the fact that a larger proportion of CD45RO+ Tregs than CD45RO+ responders expresses CLA. The proportion migrated did not appear to increase between 2 hours' and 5 hours' incubation, suggesting that T cells of both kinds migrate rapidly if they express the right receptors, and neither cell type showed much capacity to migrate across unstimulated endothelium. This latter point is intriguing given the small but significant presence of both conventional CD4+ T cells and CD4+ FOXP3+ T cells in healthy, unchallenged skin [314]. These may be cells remaining after a previous challenge, or perhaps representative of the very small proportion of cells that will migrate through unstimulated endothelium.

We extended our investigations of migration to include the CD45RA+ subsets of the Treg and responder pools and found little migration by either CD45RA+ Tregs or responder cells. Although CD45RA+ responder T cells migrated significantly less well than CD45RO+ responders, the difference in migration between the Treg subsets was not significant, despite strong trends. This may be due to donor variation. It is, however, possible to conclude that CD45RO+ Tregs are more likely to migrate through endothelium than CD45RA+ Tregs, and that therefore upon investigation of sections from human skin, all FOXP3+ cells would be expected to express CD45RO and not CD45RA.

The evidence from skin biopsies supported this. In sections of normal, unstimulated skin from 5 old and young donors, only two FOXP3+ cells expressed CD45RA. In

contrast, when stained for CD45RO, expression of this marker by FOXP3+ cells was almost universal. It seems clear that CD45RA+ Tregs do not preferentially home to skin, whereas skin-homing by CD45RO+ Tregs is a previously-observed phenomenon [315].

We found that bone marrow Tregs had a higher proportion of CD45RA+ cells within the pool compared to Tregs from peripheral blood. CD45RO+ cells were correspondingly decreased in the bone marrow. In contrast, there was no alteration in the proportions of CD45RA+ and CD45RO+ cells among responders, despite higher expression of CXCR4 cells by the naïve cells in this pool; this suggests that mechanisms other than the CXCL12-CXCR4 interaction may be causing the enrichment of CD45RA+ Tregs in the bone marrow. We investigated expression of Ki67 by all the subsets in an attempt to ascertain whether the CD45RA+ cells were simply proliferating more in the bone marrow environment, but could find no difference in the extent of proliferation in this subset.

There was a lower degree of Ki67 expression amongst CD45RO+ Tregs in the bone marrow, although no significant differences in the proliferation rates of the other Treg and responder T cell subsets. This lower proliferation rate amongst CD45RO+ meant that in bone marrow, there is no significant difference in the rate at which the two Treg subsets are cycling. This is in contrast to the blood, where CD45RO+ cells are significantly more proliferative; this observation is a reminder that the blood, while a useful and highly-accessible source of *ex vivo* cells, does not necessarily provide a representative picture of the behaviour of immune cells elsewhere in the body. The altered cycling rate amongst CD45RO+ regulatory T cells may contribute to the altered proportions of the subsets, but given their high expression of CXCR4, it seems probable that CD45RA+ Tregs are actively homing to the site. The reason for this preference is not yet clear, but it is possible that the cytokine environment of the bone marrow is conducive to the survival or development of CD45RA+ Tregs. They may also be able to proliferate in this environment without losing CD45RA expression. The bone marrow has also been demonstrated to be a secondary, as well as a primary, lymphoid organ [Feuerer et al, 2003]. It is possible that these cells are primed here.

These results show a distinct migration pattern for CD45RA+ and CD45RO+ Tregs. CD45RO+ Tregs seem likely to home to the skin. CD45RA+ Tregs, on the other hand, show a preference for the bone marrow and seem less likely to be found at sites prone to inflammation. Further investigation is required to establish the reason for this

differential migration, and whether these cells home to different environments for survival purposes or in order to fulfil different roles. Additionally, although Tregs and responders showed an equal capacity for rapid transendothelial migration when isolated, an area that remains to be investigated is the interaction between the two cell types on an endothelial monolayer. It is possible that, when allowed to interact, Tregs may affect the migration of responders; Tregs have previously been reported to interfere with the rolling and adhesion of CD4+CD25- cells *in vivo* [248].

Chapter 5. Human FOXP3+ Tregs in the immune response *in vivo*

5.1 Introduction

The previous two chapters investigated the phenotype and behaviour of regulatory T cells, and their CD45RO+ and CD45RA+ subsets, both in the blood and in two distinct tissues, the skin and bone marrow, in the normal, healthy individual. However, Tregs exist to exert a suppressive function, not only reducing the ever-present risk of autoimmunity in the periphery but also dampening immune responses once they are no longer required, in order to prevent chronic inflammation and immunopathology. To investigate whether responder T cells can convert to regulatory T cells during an immune response, we initially investigated the upregulation of FOXP3 in response to stimulation of CD4+CD25- cells *in vitro*. A large proportion of these responder T cells acquires expression of FOXP3 following stimulation. We showed, however, that this expression is purely transient, appears to be linked to proliferation, and that the cytokine profile of these activated cells does not match that of FOXP3+ Tregs, which means that study of FOXP3+ cells *in vivo,* especially in an inflammatory environment, must be undertaken with caution.

Tregs do, however, participate in immune responses *in vivo* and these primarily occur in the tissues. This chapter seeks to characterise the behaviour and migration of Tregs in the skin during the human immune response to a secondary antigen challenge, namely PPD (to which most adults have pre-existing immunity, having been vaccinated with the BCG vaccine) *in vivo*. Following secondary antigen challenge in the skin, there is an influx of lymphocytes, many of which are CD4+ and a constant proportion of which expresses FOXP3 [314]. Since FOXP3 expression can be acquired transiently by non-Tregs *in vitro*, we sought to establish whether these FOXP3+ cells were true Tregs via detailed investigation of their phenotype and cytokine production.

5.2 Acquisition of FOXP3+ expression after stimulation of non-Treg CD4+ T cells *in vitro*

Previous studies have indicated that stimulation of human CD4+CD25- T cells results not just in acquisition of expression of CD25, but also the induction of FOXP3 expression in these conventional T cells. This expression is not accompanied by suppressive function [196]. In order to further investigate the kinetics of this phenomenon, CD4+CD25- cells were isolated from peripheral blood and stained with CFSE. They were then incubated with anti-CD3, anti-CD28 coated beads as a stimulus for up to seven days. Samples were taken at day 1, day 3, day 5 and day 7 and investigated for expression of FOXP3 and CD25. We also stained for Ki67, to measure proliferation, and CFSE, to provide a proliferative history of the cells: those which have divided more have a weaker CFSE signal. As shown in Figure 5.1, FOXP3 expression preceded Ki67 expression. After 24 hours, around 10% of cells showed expression of FOXP3, but these cells did not express Ki67. FOXP3 expression peaked at day 3, by which time almost all FOXP3+ cells were Ki67+ and most showed evidence of division, both by expression of Ki67 and dilution of CFSE. FOXP3 expression then fell, until by day 7 only around 7% of the cells expressed the marker; the vast majority were, however, still dividing.

Subsequent investigation of the cytokine profiles of stimulated responder cells revealed similar proportions of both FOXP3- and FOXP3+ cells producing the pro-inflammatory cytokines IL-2 and IFN-γ (Figure 5.2). This supports the suggestion that those stimulated responder CD4 T cells which have upregulated FOXP3 expression have not acquired a regulatory phenotype. The transient but widespread observed expression of FOXP3 makes analysing activated cells difficult. Steps must be taken to ensure that FOXP3+ T cells in an inflammatory environment are true Tregs.

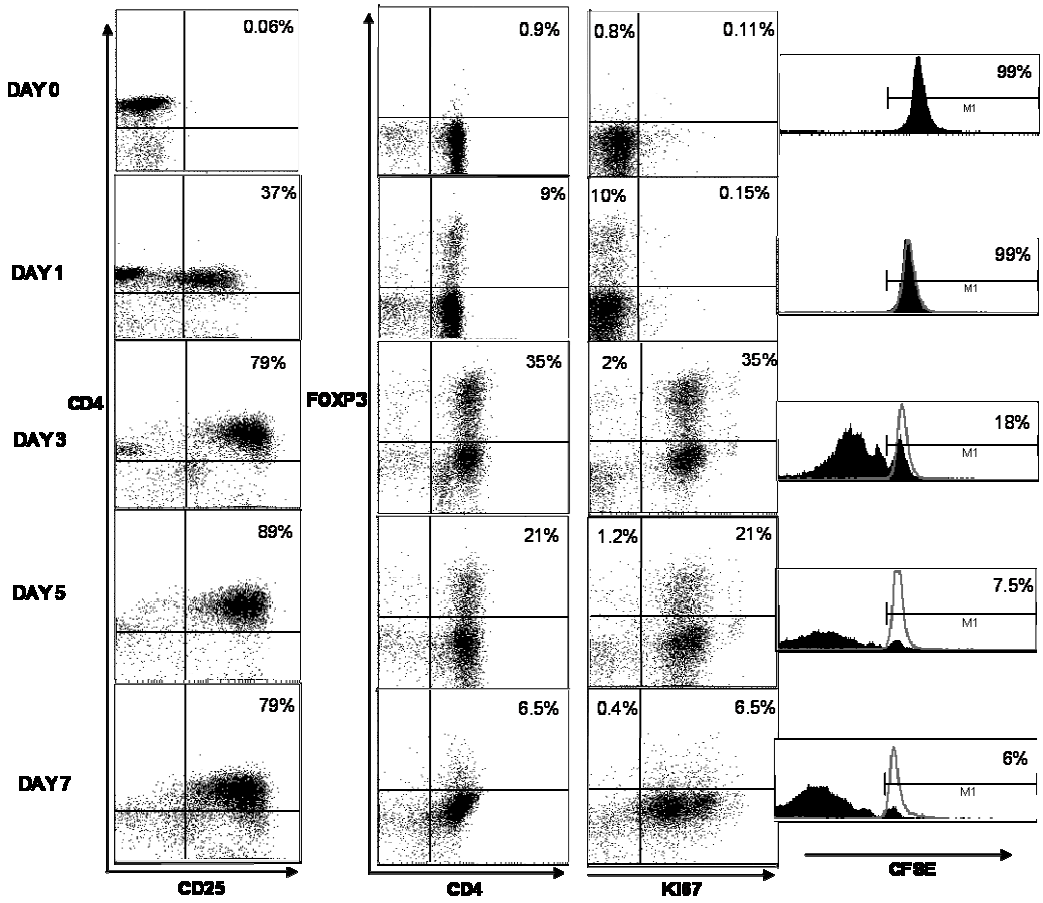


Figure 5.1. Acquisition of FOXP3 expression by stimulated cells.

CD4+CD25- cells were isolated from peripheral blood and stained with 0.5µM CFSE. They were incubated with anti-CD3, anti-CD28-coated beads for seven days, with samples taken at day 1, day3, day 5 and day 7 and stained for expression of CD25, FOXP3 and Ki67. Dilution of CFSE indicates cell divisions have occurred.

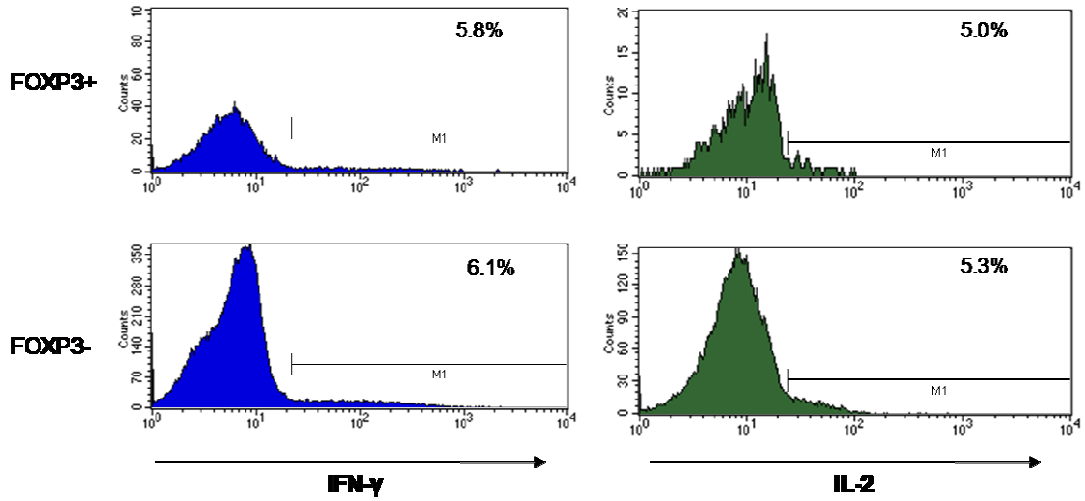


Figure 5.2. Cytokine production by FOXP3-expressing stimulated responder CD4 T cells.

CD25- responder T cells were isolated and stimulated *in vitro* with anti-CD3- and anti-CD28 coated beads. After 3 days the cells were fixed and stained for production of pro-inflammatory cytokines. Histograms show IFN-γ and IL- production 2 by the resultant FOXP3+ (top) and FOXP3- (bottom) cells.

5.3 Accumulation of FOXP3-expressing cells in human skin following secondary antigen challenge

In order to investigate the kinetics of FOXP3+ T cell accumulation during a secondary immune response, skin punch biopsies were taken at days 0, 3, 7 and 14 following subcutaneous injection of the PPD antigen, a component of the BCG vaccine. As shown in Figure 5.3D, the proportion of CD4+ T cells expressing FOXP3 remained the same throughout the immune response. Expression of Ki67 by FOXP3+ cells remained relatively constant throughout the course of the immune response (Figure 5.4), at around 8-9% (mean day 3: 9.7%±1.1; day 7: 8.1%±2.5; day 14: 7.9%±1.8).

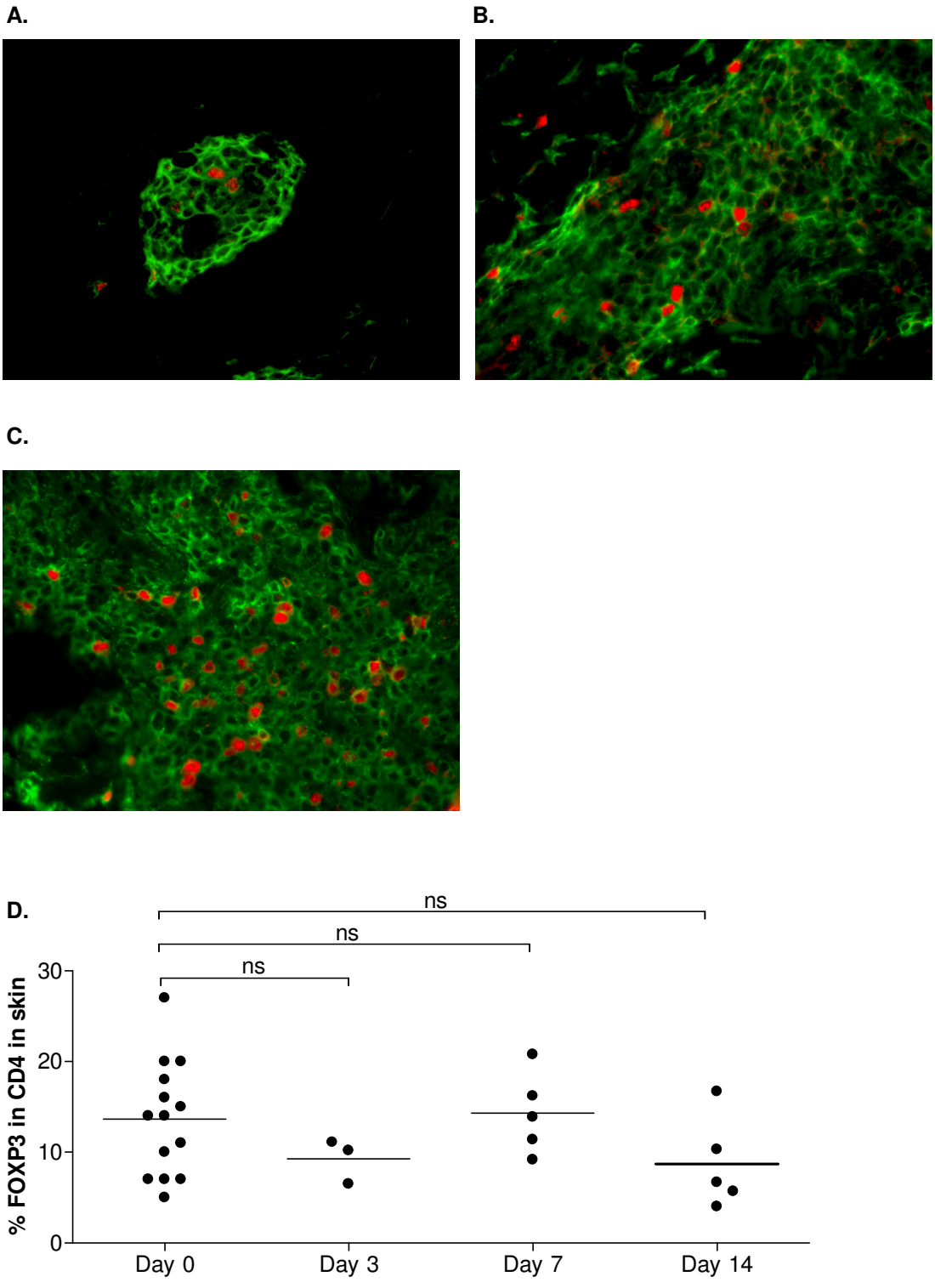


Figure 5.3. FOXP3+ cells accumulate at the site of antigen (PPD) challenge.

Healthy volunteers were subcutaneously injected with PPD antigen and punch biopsies taken at the injected site at various different timepoints. Sections from these biopsies were stained for CD4 and FOXP3. Representative pictures are shown from **A.** day 0, **B.** day 3 and **C.** day 7 sections, showing CD4 (green) and FOXP3 (red) expression in unchallenged and inflamed skin. **D.** Graph showing the proportion of CD4+ cells expressing FOXP3 during the course of the immune response.

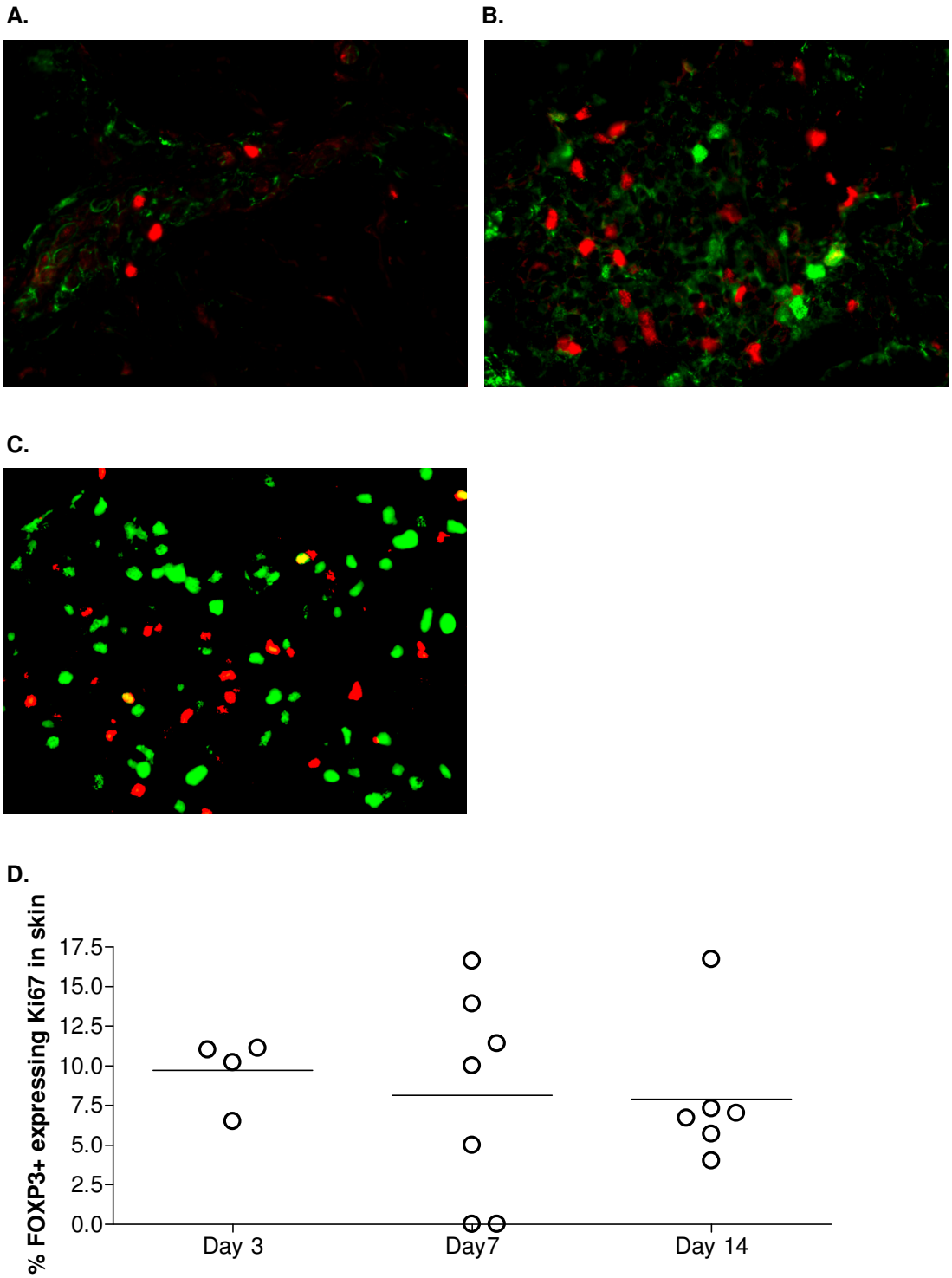


Figure 5.4. FOXP3+ cells proliferate in the skin following subcutaneous secondary antigen challenge.

Healthy volunteers were subcutaneously injected with PPD antigen and punch biopsies taken, before sections were stained for expression of FOXP3 and Ki67. Representative pictures are shown from **A.** day 0, **B.** day 3 and **C.** day 7 showing expression of Ki67 (green) and FOXP3 (red) following antigen challenge. **D.** Graph showing expression of Ki67 by FOXP3+ cells (FOXP3+ and Ki67+ cells are yellow) at day 0, day 3, day 7 and day 14 after antigen challenge.

5.4 Phenotypic analysis of FOXP3+ cells accumulating in the skin after a secondary antigen challenge

There is a clear increase in the absolute number of CD4+FOXP3+ T cells present at the site of an immune response as the response grows in magnitude, up to a peak at day 7, as well as an increase in their proliferation. However, the proportion of CD4+ T cells at the site expressing FOXP3 does not increase: the number of FOXP3+ T cells becomes larger in line with the total number of CD4+ T cells. It is not clear whether these FOXP3-expressing cells really are Tregs or simply activated CD4 T cells. This study sought to establish this, by investigating the expression of known Treg markers and examining the behaviour of the FOXP3+ and FOXP3- cells at the site. This was achieved with the suction blister technique [313, 314], whereby a blister is formed over the site of antigen challenge and cells and fluid allowed to accumulate within it for 18 hours. Cells from the immune reaction move into the blister fluid during this time. The fluid is then extracted from the blister and the cells within it purified. They can then be analysed by flow cytometry.

5.4.1 CD4+FOXP3+ cells following PPD challenge have a Treg phenotype

We induced suction blisters over the site of PPD challenge, 7 days after subcutaneous antigen injection, and stained for FOXP3 and CD4 as well as CD25, CD127 (the IL-7 receptor α-chain), CD39 and CD27 – all markers of which Tregs are thought to have a specific expression pattern. Figure 5.5 shows representative staining of the blister FOXP3+ cells for these markers; they are $CD25^{hi}$, almost entirely CD27+, largely CD39+ and also $CD127^{lo}$, which are all recognised characteristics of human Tregs. In comparison, staining of FOXP3- cells, also shown in Figure 5.5, revealed some expression of CD25 but lower CD27 and CD39 expression, as well as high expression of CD127. This is a typical profile of an activated T cell.

In addition to the Treg markers mentioned above, expression of Ki67 by the CD4+ blister cells was also investigated. Ki67 expression by FOXP3+ cells in blisters was not significantly different from that in the peripheral blood (Figure 5.6), but Ki67 was expressed by a mean of 28.8%±10.1 of FOXP3- cells at the site of the immune response compared with a mean of 1.7%±0.26 in the peripheral blood – a significant increase.

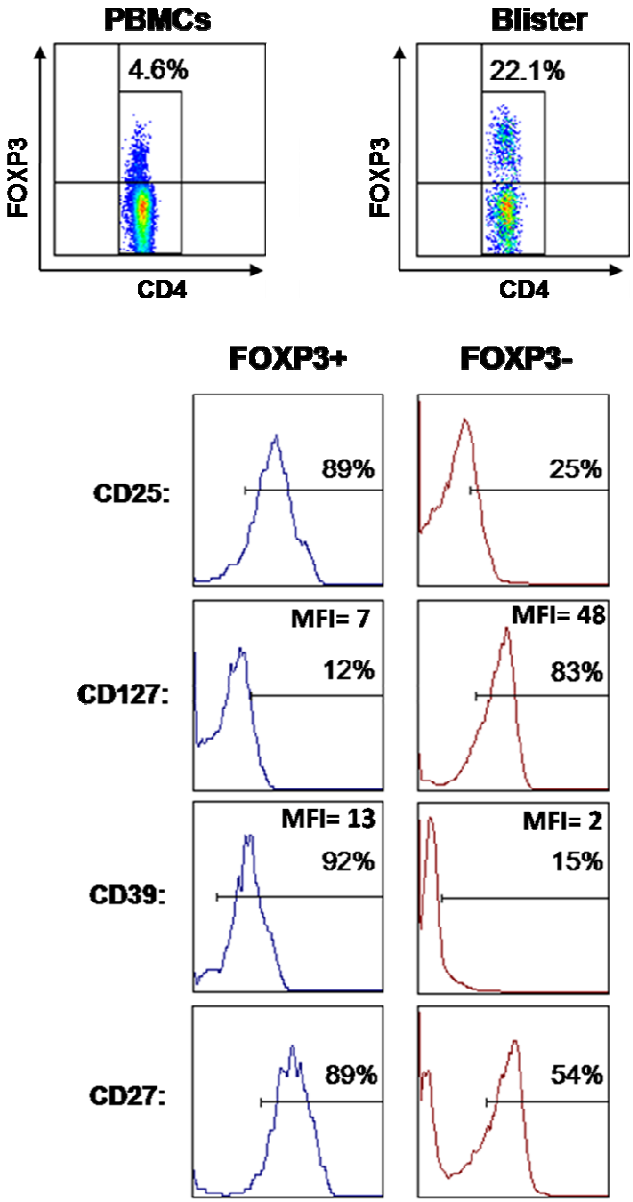


Figure 5.5. FOXP3+ cells at the site of an immune response in vivo have regulatory T cell-like phenotypes.

Suction blisters were formed over the site of a day 7 cutaneous response to PPD and the cells were harvested and stained with monoclonal antibodies against the Treg markers FOXP3, CD25, CD127, CD27 and CD39. Data are representative of six independent experiments.

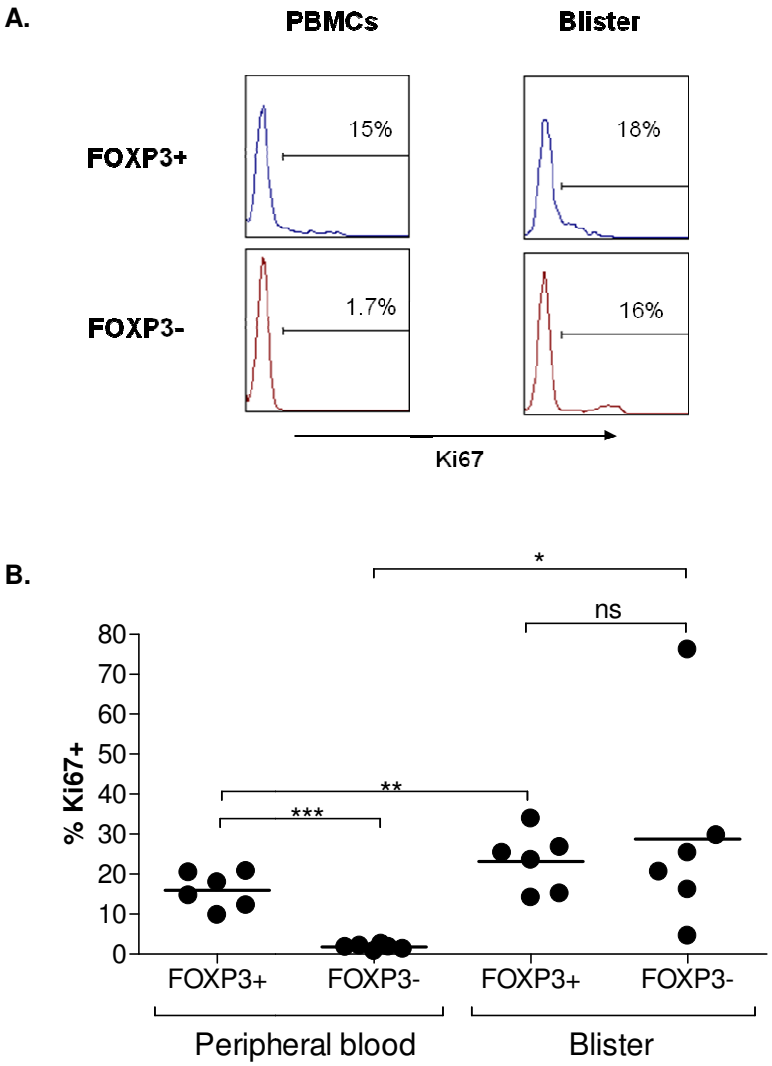


Figure 5.6. FOXP3+ and FOXP3- CD4+ cells at the site of an ongoing secondary immune response are proliferating.

Cells were isolated from a suction blister formed over a day 7 secondary cutaneous response to PPD. They were stained for expression of CD4 and FOXP3, and the proliferation marker, Ki67, as well as the skin-homing marker, CLA. Data are representative of six independent experiments.

5.4.2 Accumulated CD4+FOXP3+ T cells do not produce pro-inflammatory cytokines in response to a PPD stimulus

Although the FOXP3+ cells accumulated at the site of antigen challenge strongly resemble Tregs phenotypically, it was still possible that they could be merely activated responder (FOXP3-) cells. The ideal test of whether these cells were Tregs would be to test their suppressive capacity, but due to the small numbers of cells involved, this proved impossible. (Commonly as few as 100,000 cells can be retrieved from a skin blister, of which 40,000 express CD4 and a far smaller proportion express FOXP3.) Instead, we harvested the total cells from the blister and restimulated them with PPD before staining them to detect cytokine production: Tregs should not produce any IL-2 or IFN-γ in response to stimulation. We found no cytokine production by FOXP3+ cells isolated from the blister, in comparison to high levels of IFN-γ and IL-2 produced by the FOXP3- CD4+ population (Figure 5.7). The putative Tregs also produced little or no IL-17 and IL-10.

A.

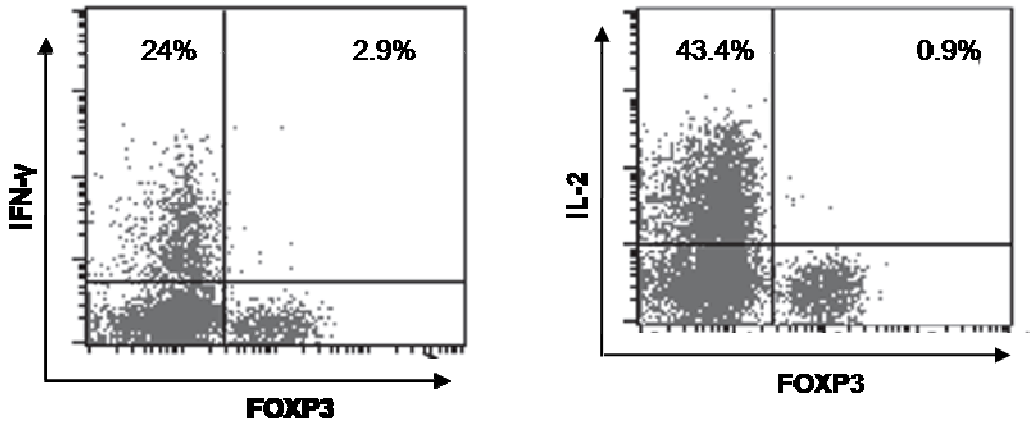


Figure 5.7. FOXP3+ cells at the site of an ongoing cutaneous immune response do not produce cytokines.

Cells were extracted from suction blisters raised over a cutaneous response to PPD antigen. They were stimulated with PMA and ionomycin, then exocytosis was blocked using brefeldin A. Cells were stained for expression of CD4, FOXP3 and the cytokines IFNγ and IL-2. Data is representative of three independent experiments. *Left.* Production of IFN-γ by FOXP3+ and FOXP3- blister cells within the CD4+ gate. *Right.* Production of IL-2 by FOXP3+ and FOXP3-blister cells within the CD4+ gate.

5.5 Discussion

Work in this chapter investigates the response of Tregs in an inflammatory environment. *In vitro*, it is clear from work in this chapter and also from previous studies [193-196] that stimulated effector CD4 T cells express FOXP3 to relatively high levels, and this expression seems to be linked to proliferation since – apart from on day 1 – all FOXP3+ cells are also Ki67+. The appearance of FOXP3 before the first expression of Ki67 is a curious phenomenon given the fact that Tregs are also generally accepted to be anergic *in vitro* [182, 183, 187], and the ectopic expression of FOXP3 also causes anergy in the transformed cells [157-159, 197]. It would seem that the upregulation of FOXP3 in response to stimulation does not, however, prevent proliferation or cytokine production. FOXP3 expression in an inflammatory environment *in vitro* cannot be taken as a marker for Tregs.

In vivo, however, the situation may be different: FOXP3+ cells appear to be both proliferative and true Tregs. We investigated the response of CD4+ T cells to a secondary cutaneous challenge, subcutaneous injection of PPD, initially by immunofluorescent staining of skin sections. We observed the number of CD4+FOXP3+ cells increasing along with the number of CD4+FOXP3- T cells. The proportion of FOXP3+ cells stayed the same throughout the course of the response, as did their degree of proliferation.

In order to investigate whether these FOXP3+ cells were really Tregs or activated immune cells, we formed suction blisters over the area of immune response; the immune cells moved into the blister fluid, which was then extracted so that the cells could be analysed by flow cytometry. We stained the cells extracted for several different Treg markers, including FOXP3 and CD127 (IL-7Rα), as well as a marker of proliferation (Ki67) and a marker of differentiation (CD27). We found that the FOXP3+ and FOXP3- cells from the blister had very different phenotypes; the FOXP3+ cells expressed high levels of the Treg-associated markers CD25 and CD39 and low levels of CD127, a classic Treg phenotype [181, 183, 212, 213, 216], whereas the FOXP3- cells had the opposite phenotype. Both FOXP3+ and FOXP3- cells largely expressed CD27, although the FOXP3+ cells expressed more. Tregs are known to be almost universally CD27+ [343]. Curiously, Ki67 was expressed by the FOXP3+ cells to approximately the same degree as those isolated from the peripheral blood, but FOXP3- cells were proliferating much more than Tregs at the site of the immune response.

The phenotypes described above suggest strongly that the FOXP3+ cells found at the site of an *in vivo* immune response are regulatory T cells. However, the fact that the FOXP3+ cells isolated from blisters do not produce pro-inflammatory cytokines is stronger evidence. FOXP3+ cells isolated from an *in vivo* immune response do not produce notable levels of either IFN-γ or IL-2; if they were simply activated cells they would be expected to produce very high levels of one or both of these cytokines, as the FOXP3- cells from the blister do. The strongest possible evidence would come from isolating these cells and demonstrating that they suppress. However, often only 100,000 cells can be harvested from a blister. Of these, around 40-60% are CD4+ and around 10-20% of the CD4+ cells express FOXP3. This number of cells is too low to pass through magnetic columns and obtain a usable yield of Tregs; we attempted to pan the cells, using 96-well plates coated with anti-CD25 and anti-CD39 antibody, but could not achieve the required purities for a suppression assay of any kind via this method. The number of cells was so low that sorting them would have resulted in many dying, if indeed pure populations could be obtained by sorting such a small number of cells. However, the lack of cytokine production by cells coming from such an inflammatory environment strongly suggests that these skin-derived FOXP3+ cells are indeed Tregs.

These studies raise interesting points about the use of FOXP3 as a marker *in vitro* and *in vivo*. In *in vitro* conditions it is clear that FOXP3 is an activation marker in conventional T cells; however, in the highly-inflammatory conditions of a secondary immune response, FOXP3 only seemed to be expressed by cells which appeared to be regulatory T cells. It is plausible that FOXP3 is expressed after stimulation only in the absence of the complex cytokine environment found *in vivo*, suggesting that its use as a definitive human Treg marker for *in vivo* and *ex vivo* work is valid. Additionally, the cells were stimulated using beads coated in anti-CD3 and anti-CD28, which is an extremely strong stimulus – far stronger than would be encountered *in vivo*.

Another question raised by the observations made in this chapter is the source of the FOXP3+ cells at the site of inflammation. The cells are certainly undergoing proliferation throughout the course of the immune response, as seen by their high expression of Ki67 in both the blisters and the immunofluorescently-stained biopsy sections, but the expansion of FOXP3+ cells could also result from conversion of conventional CD4 T cells. If conversion can occur as a result of anergy, as suggested in Chapter 3, then the dense T cell environment of a secondary delayed type hypersensitivity (DTH) response could be conducive to this. Many T cells clustered

together provide the perfect conditions for T:T presentation and consequent anergy induction. This would also generate antigen-specific Tregs, which could specifically protect against pathology caused by any further immune responses against this antigen. Further work is required to definitively ascertain the mechanism of the Treg expansion. New markers, such as the Treg-specific demethylated region (TSDR) [218] and the transcription factor, Helios [344], may be able to aid in establishing whether these cells result from expansion of pre-existing Tregs or conversion of conventional T cells at the site of inflammation.

Chapter 6. Human FOXP3+ Tregs and atopic dermatitis

6.1 Introduction

Atopic dermatitis, also known as atopic eczema, is a hypersensitivity condition that is growing in prevalence in the developed world [317, 318] and causes inflammation of the skin with associated itching and lichenification [321]. It is commonly seen in children, and frequently resolves itself as they mature [316, 318]. It is known to be a multifactorial disease [321, 323, 324], but Tregs may have a role to play in its pathogenesis, since eczema-like symptoms are frequently seen in the human autoimmune condition IPEX, in which FOXP3 is mutated and non-functional [184, 185, 327], as well as the murine equivalent of the disease, known as scurfy [184, 185]. Other studies have also implicated Tregs in atopic dermatitis [328, 332]. This study aimed to delineate the possible role of Tregs in atopic dermatitis (AD) by investigating differences in the phenotypic and functional properties of AD Tregs, compared to those from healthy controls. We investigated total Tregs and also the naïve and memory Treg subsets.

The severity of the condition experienced by AD donors was measured using the EASI score [345, 346]. This assessment is performed by a trained dermatologist and allocates each component of the condition (for example, redness, lichenification and papulation), in each section of the body, a number – depending on the severity, from 1-3. The EASI method also takes into account the spread of AD over the body by incorporating an ‘affected area score’. EASI scores in the patients studied ranged from the relatively mild (lowest score 0.8) to very severe (highest score 57.0).

6.2 Proportions of Tregs in atopic dermatitis

In order to establish whether or not there is a deficit in the proportion of total Tregs, or the CD45RA+ and CD45RO+ subsets within the FOXP3 pool, peripheral blood was harvested from AD patients at a range of severities (EASI 1.9-57), and age-matched healthy controls. PBMCs were stained for expression of CD4, FOXP3, CD45RA and CD45RO. Although there was no difference between AD patients and healthy controls in the total proportion of peripheral blood CD4+ T cells expressing CD25 to a high level (Figure 6.1A; p=0.675, Mann-Whitney U Test), within the Treg pool, AD patients had a significantly decreased proportion of CD45RA+ Tregs compared to age-matched controls (p=0.04, Mann-Whitney U Test). This difference among Tregs was not reflected among conventional FOXP3- CD4+ T cells, where the proportion of CD45RA+ cells was no different to age-matched controls. There was a strong negative correlation between the proportion of Tregs expressing CD45RA and the severity of disease as measured by the EASI score (Figure 6.1B; p=0.003).

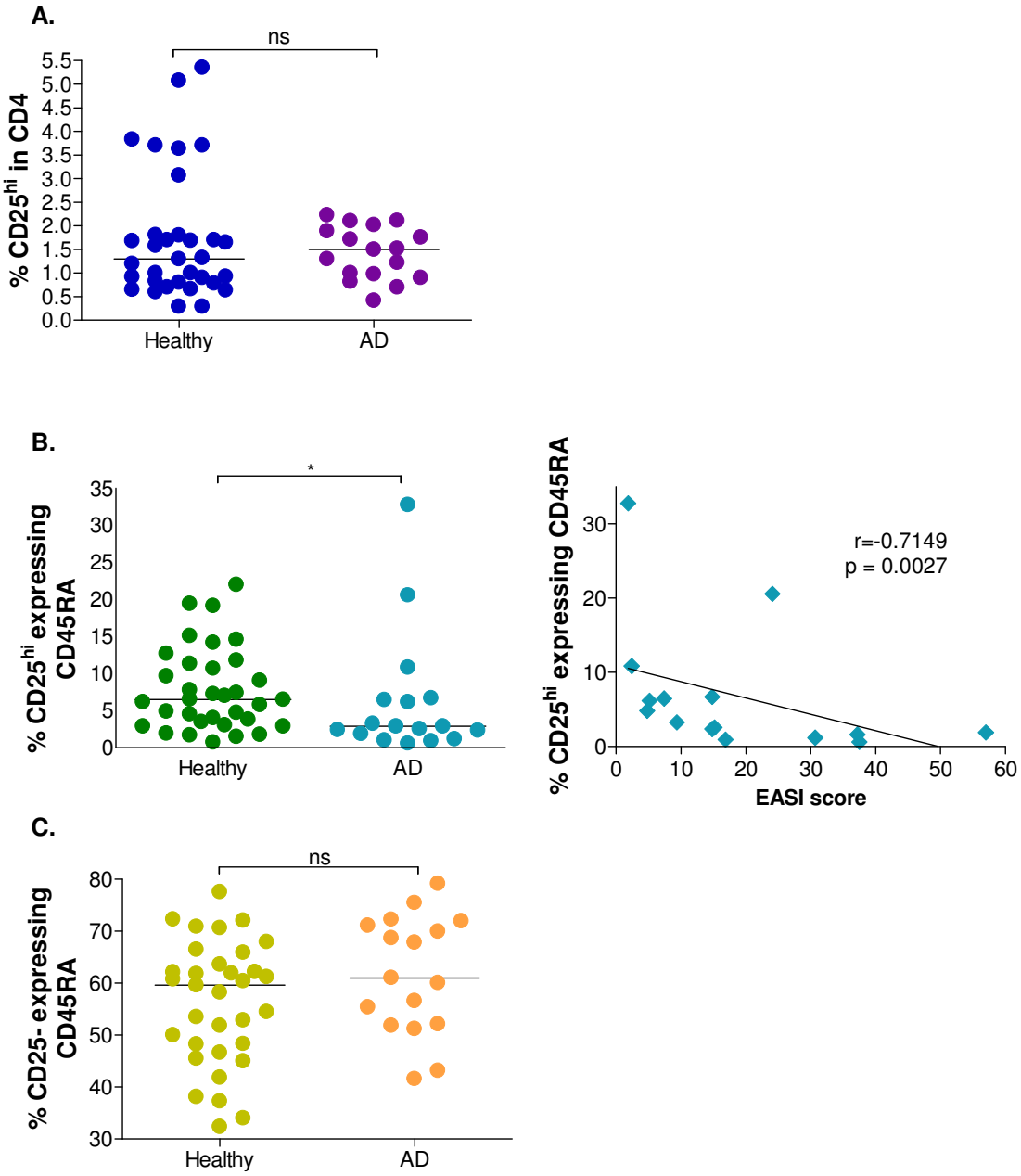


Figure 6.1. Composition of the CD4+, Treg (CD25^{hi}) and responder pools in healthy volunteers and patients with atopic dermatitis.

A. Proportion of CD25^{hi} cells in the CD4+ T cell subset from the peripheral blood of healthy controls and age-matched AD patients (n=33 healthy, 17 AD). **B.** *Left*: proportion of CD25^{hi} CD4+ T cells expressing CD45RA in healthy controls and AD patients (n=33 healthy, 17 AD). *Right:* graph showing correlation between CD45RA expression in CD25^{hi} and EASI score in patients with atopic dermatitis (n=12). **C.** Proportion of CD4+ responders expressing CD45RA in healthy controls and AD patients (n=33 healthy, 17 AD).

6.3 Proliferation of AD Tregs

Having established a significant difference in the composition of the Treg pool in AD patients, we next sought to establish whether there was any difference in the *ex vivo* degree of proliferation, as indicated by expression of the nuclear protein, Ki67. We harvested PBMCs from the peripheral blood of AD patients and stained them for CD4, FOXP3, Ki67 and CD45RO, comparing expression of Ki67 with that among age-matched healthy controls. Surprisingly, we found that although there was no difference in proliferation of the CD45RO+ Treg subset, there was a significantly lower degree of proliferation among CD45RA+ Tregs in AD patients than in healthy controls (healthy: mean 1.6%±0.2, n=9; AD: mean 0.41%±0.18, n=5; p=0.0039). There was also a greater degree of proliferation among CD45RO+ responder T cells in AD patients than in healthy controls; this may be as a result of the chronic inflammation of the tissues in these patients.

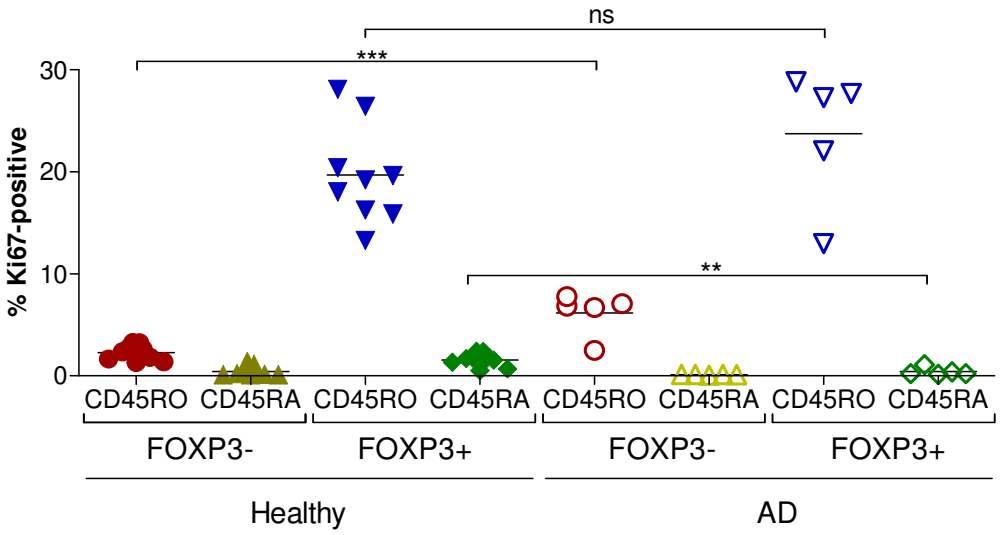


Figure 6.2. Ki67 expression by T cell subsets in the peripheral blood of AD patients is altered from that in healthy controls.

PBMCs from AD patients and age-matched healthy controls were stained *ex vivo* for the Treg markers CD4 and FOXP3, the differentiation marker CD45RO and the proliferation marker Ki67. Cumulative data is shown (healthy: n=9, AD: n=5).

6.4 Phenotypes of AD Tregs

Having established a deficit in CD45RA+ Treg numbers in AD patients and also a discrepancy in their rate of proliferation, we sought to further characterise any potential functional defects in the CD45RA+ Treg population by investigating their expression of the markers CTLA-4 and CD39.

In the case of CD39, no significant difference was found in its expression overall in either Treg subset (Figure 6.3B and C). However, there were two distinct groups of patients, expressing either normal levels of CD39 or much lower levels, among CD45RO+ Tregs. CD39 expression did not, however, correlate with the severity of the AD (Figure 6.3B, right).

No difference was observed in intracellular CTLA-4 expression by CD45RA+ Tregs (Figure 6.4 A and B). However, a significantly increased proportion of CD45RO+ Tregs expressed CTLA-4 in AD patients compared to healthy controls (mean healthy 12.1%±1.2, n=7; mean AD 24.0%±3.9, n=8; p=0.0173). CTLA-4 expression did not significantly correlate with CD39 expression on Tregs, either from healthy or AD individuals (Figure 6.4C).

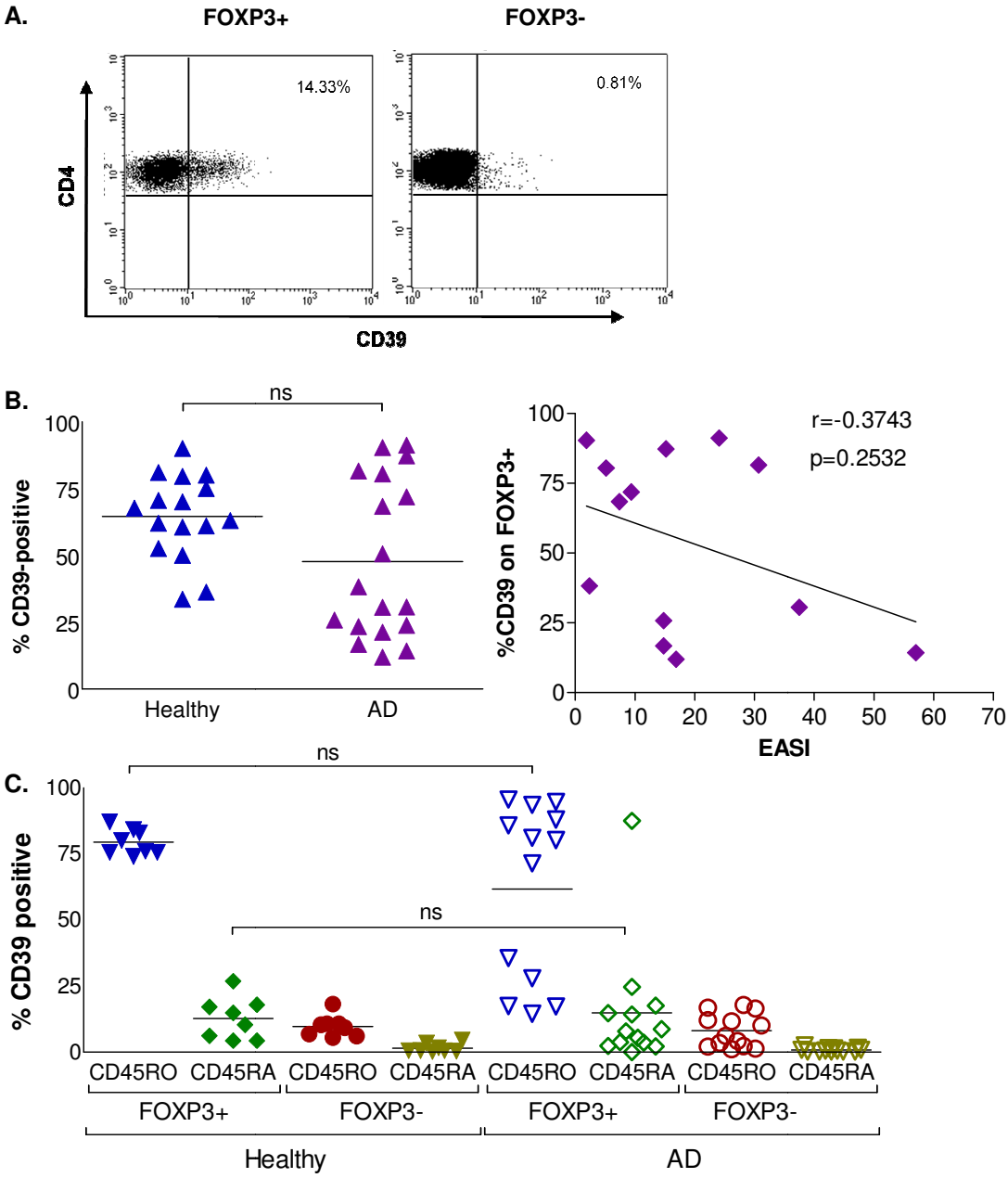


Figure 6.3. CD39 expression on Tregs and responder T cells from AD patients in comparison with healthy controls.

A. Dotplots showing representative CD39 staining on Tregs (left) and responders (right) from AD patients. **B.** *Left,* CD39 expression by CD4+FOXP3+ Tregs in healthy controls and AD patients. (n=16 healthy, 18 AD) *Right,* graph showing CD39 expression by FOXP3+ Tregs compared to eczema severity (EASI score) in AD patients (n=13). **C.** Cumulative data showing CD39 expression by CD45RO+ and CD45RA+ subsets in healthy controls and AD patients. (n=8 healthy, 13 AD)

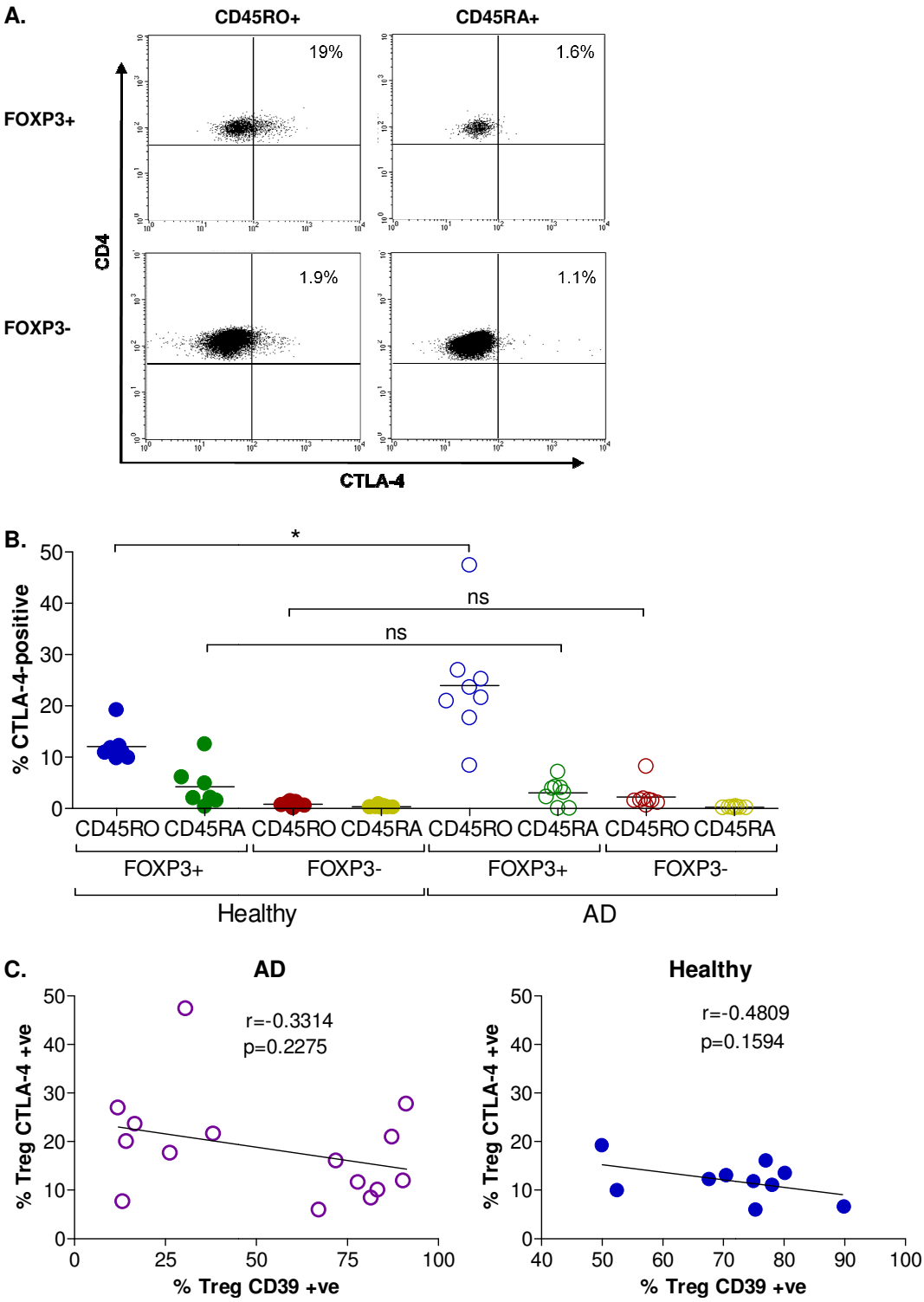


Figure 6.4. CTLA-4 expression on Tregs and responders from AD patients in comparison with healthy controls.

A. Dotplots showing representative intracellular staining of CTLA-4 on Treg and responder subsets from AD patients. **B.** Cumulative data showing intracellular CTLA-4 expression by healthy and AD CD45RA+ and CD45RO+ T cell subsets. (n=7 healthy, 8 AD) **C.** *Left:* Graph showing CTLA-4 expression compared with CD39 expression on Tregs of AD patients. (n=15) *Right:* Graph showing CTLA-4 expression compared with CD39 expression on Tregs of healthy controls. (n=10)

6.5 Functional suppressive capacity of Tregs in AD

There does not appear to be a large phenotypic difference between the Tregs of AD patients and healthy controls. However, many studies have implicated defective Tregs in the pathogenesis of AD [328, 329, 332], and it is possible that they lack suppressive function. We therefore investigated functional defects in cells from AD patients– specifically for a lower capacity to suppress the *in vitro* responses of responder T cells. Cell numbers did not allow us to investigate suppression by CD45RA+ AD Tregs; we instead tested the suppressive capacity of total AD Tregs, looking at suppression of both proliferation and release of pro-inflammatory cytokines.

6.5.1 Suppression of proliferation

PBMCs were harvested from fresh blood from healthy controls and AD patients, simultaneously, and CD25^{hi} Tregs and CD25- responders isolated and incubated in a standard suppression assay set-up at a ratio of 1:1. No significant difference was observed in the suppression of AD responders by AD Tregs, compared to that of healthy responders by healthy Tregs (Figure 6.5). Although only two experiments were performed, this supports previous data obtained by our group [334].

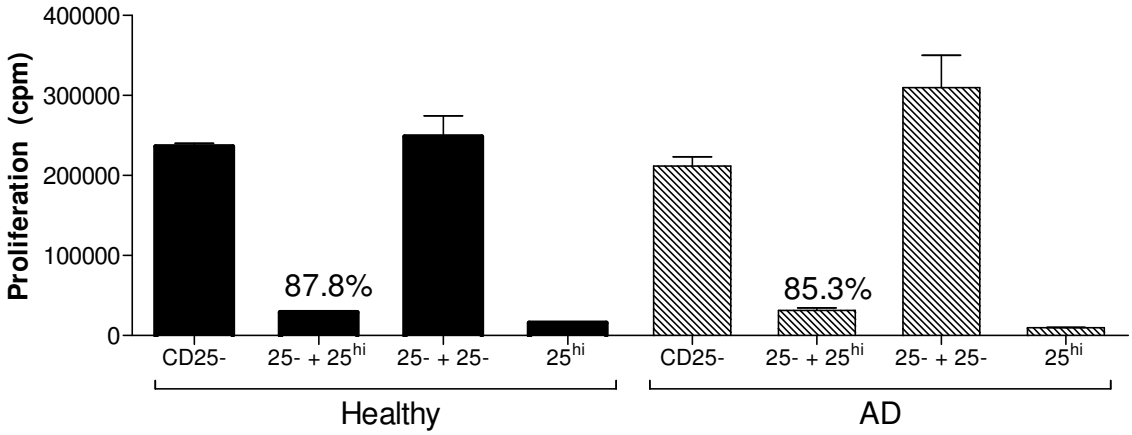


Figure 6.5. AD and healthy Tregs are equally capable of suppressing proliferation of responder T cells.

Representative graph showing results of suppression assays performed in triplicate with healthy responder T cells and Tregs (black bars) and AD responder T cells and Tregs (hashed bars). Graph is representative of two independent experiments.

6.5.2 Suppression of cytokine production

Proliferation is only one measure of a T cell response to stimulation: activated CD4+ T cells can release a range of cytokines, many pro-inflammatory, including interferon gamma (IFN-γ) and interleukin-2 (IL-2). In order to investigate whether AD Tregs have a defect in their capacity to suppress the release of inflammatory cytokines, cytokine suppression assays were set up with Tregs and responders at a ratio of 1:1. Cells from AD patients and healthy controls were investigated at the same time and cells were stained for production of the cytokines IL-2, IFN-γ, IL-4 and TNF. No difference between AD and healthy Tregs was found in suppression of any cytokine tested, although we did not observe any suppression of TNF production, by either healthy donors or AD patients (Figure 6.6).

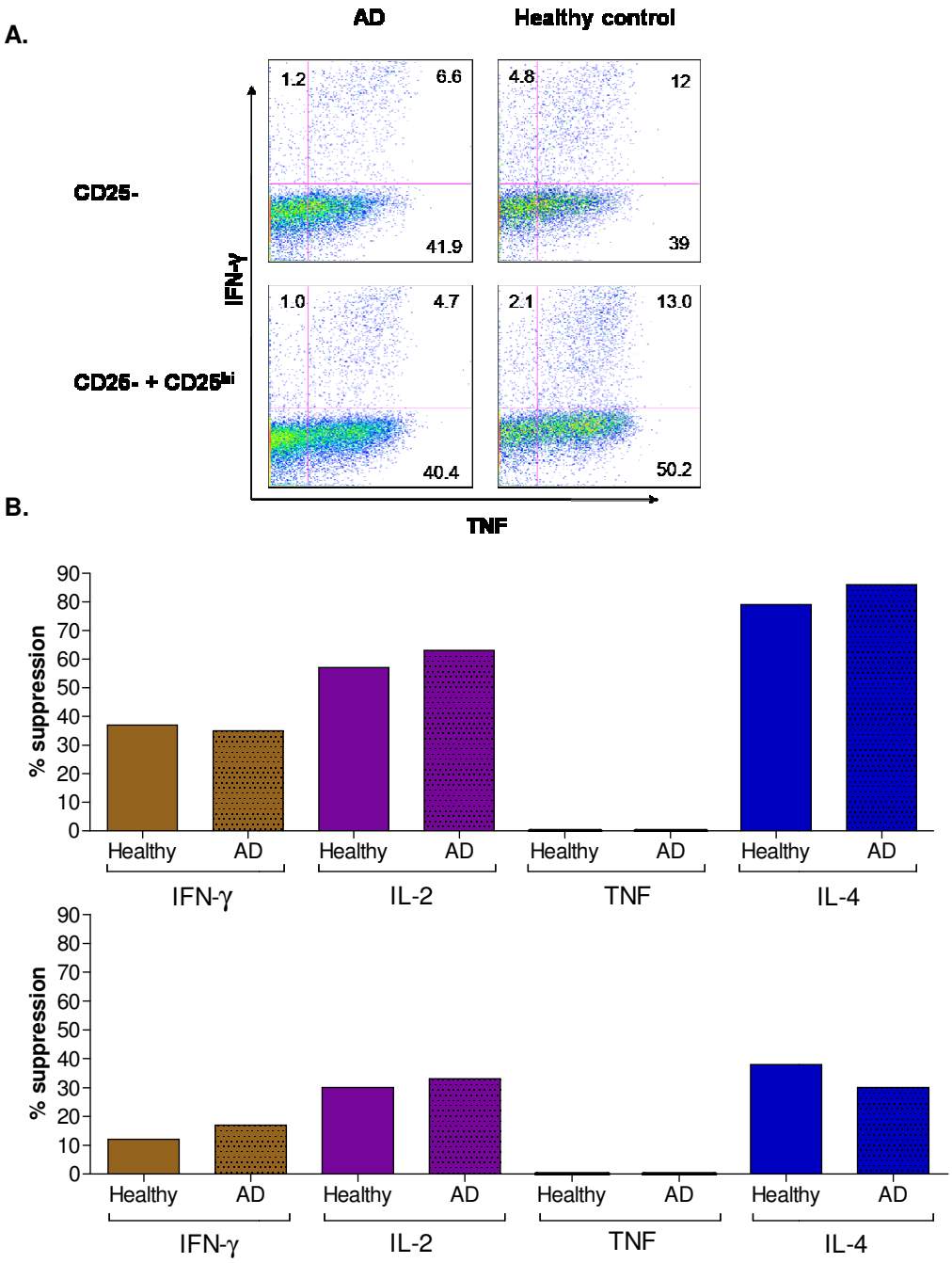


Figure 6.6. AD and healthy Tregs are equally capable of suppressing cytokine production.

CD4+CD25^{hi} and CD4+CD25- T cells were isolated from AD patients and age-matched healthy controls; responders were stained with CFSE, to differentiate them from the Tregs, and the subsets were incubated together or separately with anti-CD3, anti-CD28-coated beads for 42 hours. They were restimulated with PMA and ionomycin and secretion was blocked with brefeldin A. **A.** Representative staining showing TNF and IFN-γ production by responders alone (top) and when cocultured with CD25^{hi} Tregs (bottom) from AD patients compared to healthy controls. **B.** Cytokine production in each subset was measured using flow cytometry. Two independent experiments are shown.

6.6 Skin homing of Tregs in atopic dermatitis

Although total Tregs from AD patients appear to be functionally indistinguishable from their healthy counterparts *in vitro*, Tregs may nevertheless be implicated in AD *in vivo*, functioning less well in certain cytokine environments, or at low Treg:Teffector ratios, than healthy Tregs. There is also the possibility that they may be entirely functionally competent but have impaired migratory capacity, leaving them unable to reach areas of inflammation in the skin. In the final part of this study, we investigated the migration of AD Tregs, looking at their homing marker expression and their presence in the skin of AD patients *in vivo*.

6.6.1 AD Tregs show a similar degree of CLA expression to Tregs from healthy controls

CLA is a skin-specific homing marker [306], which binds E-selectin on the surface of activated endothelium [119, 120] and is known to be expressed by a large proportion of memory Tregs in healthy individuals ([314, 315]; Chapter 4). No difference in the proportion of either CD45RA+ or CD45RO+ Tregs expressing CLA was observed in AD patients compared to healthy controls, as shown in Figure 6.7. This suggests that the capacity of Tregs from AD patients to reach the inflamed site is unimpaired (healthy: mean 42.7%±4.7 CD45RO+ Tregs, 16.4%±4.7 CD45RA+ Tregs, n = 12; AD: mean 49.7%±7.7 CD45RO+ Tregs, 11.7%±2.8 CD45RA+ Tregs, n = 6).

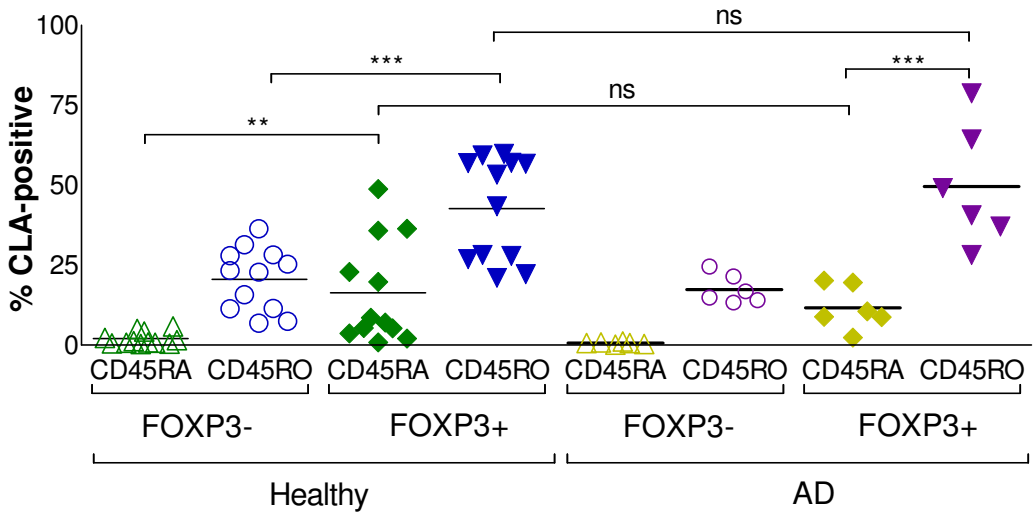


Figure 6.7. Similar CLA expression by Tregs and non-Tregs in atopic dermatitis patients and healthy controls.

Peripheral blood mononuclear cells from AD patients and age-matched controls were stained for expression of CD4, FOXP3, CD45RA, CD45RO and CLA. CLA expression by T cell subsets was compared in the two groups. No significant difference in CLA expression between healthy and AD subjects was found in any CD4+ T cell subset.

6.6.2 CD4+ FOXP3+ cells are present in the skin of AD patients

Punch biopsies were taken from the lesional and nonlesional skin of AD patients (n=7 lesional, 8 nonlesional) and sections were stained for CD4 and FOXP3. CD4+ cells in the five largest infiltrates in each section were counted and the proportion expressing FOXP3 compared to that in unchallenged skin sections from healthy donors. Contrary to previous reports [103], FOXP3+ cells were seen in AD sections (Figure 6.8B, C). The proportion of CD4+ cells expressing FOXP3 in both lesional and nonlesional AD skin was higher than that in normal, uninflamed skin (Figure 6.8D and see Chapter 5) and this was significant in nonlesional AD skin (mean 24.5%±2.9; unchallenged normal skin: mean 13.64%±1.7, see Chapter 5; p=0.0024), although not in lesional skin (mean proportion of CD4+ expressing FOXP3 20.2%±3.4; p=0.068). There was, however, no significant difference between the proportions of CD4+FOXP3+ cells in lesional compared to nonlesional AD skin (p=0.35).

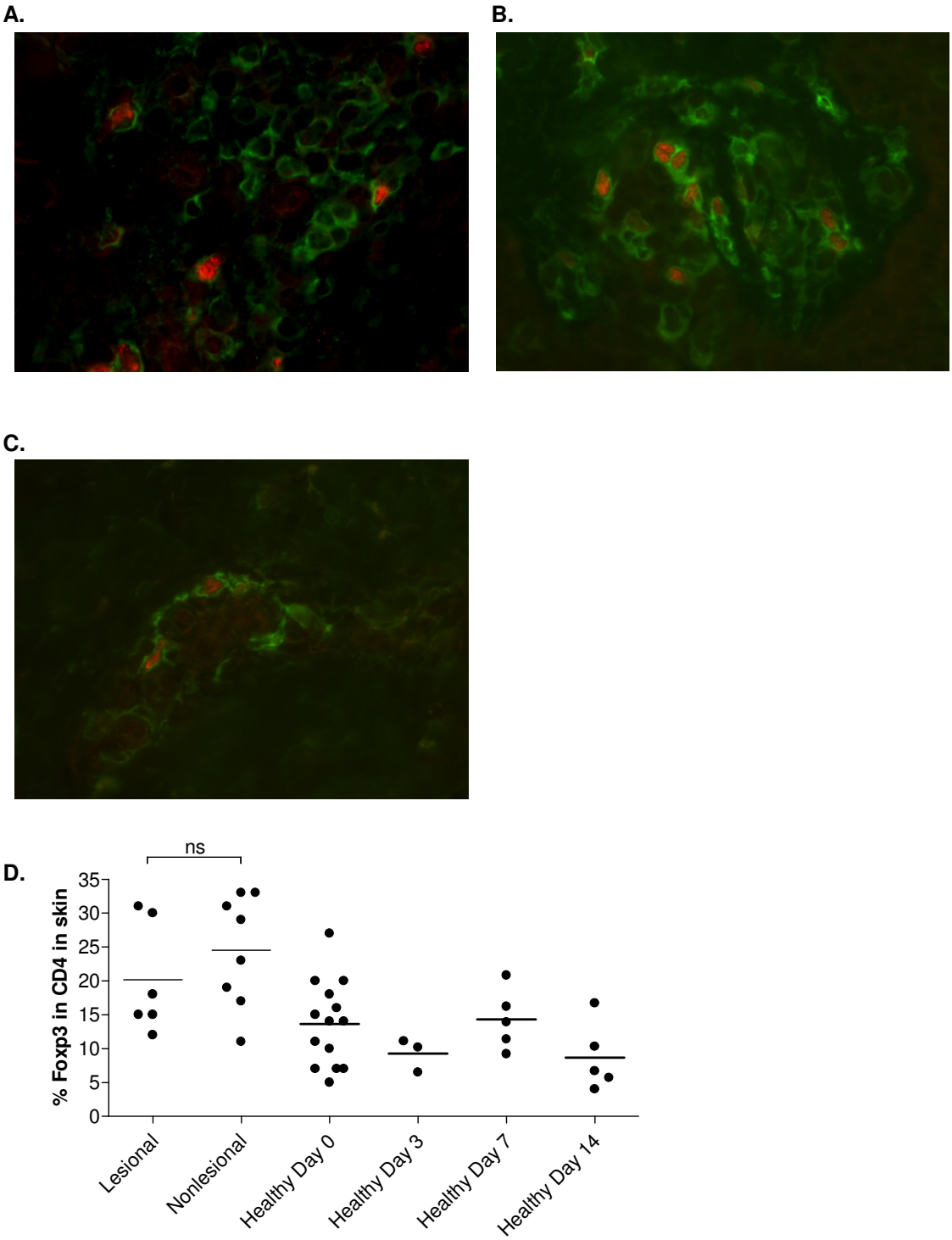


Figure 6.8. FOXP3+ cells are present in skin from atopic dermatitis patients.

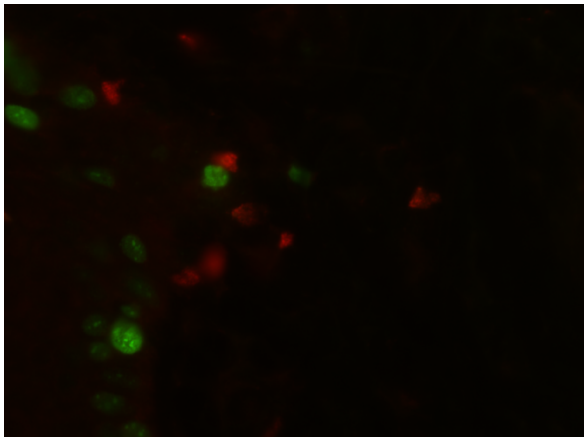
Punch biopsies were taken from healthy controls and lesional and nonlesional AD skin, and sections stained for CD4 and FOXP3. **A.** Representative picture showing expression of CD4 (green) and FOXP3 (red) in normal, unchallenged skin. **B.** Representative picture showing expression of CD4 and FOXP3 in lesional AD skin. **C.** Representative picture showing expression of CD4 and FOXP3 in nonlesional AD skin. **D.** Graph showing proportions of CD4+ cells expressing FOXP3 in lesional and nonlesional AD skin sections (n= 8 lesional, 7 nonlesional). Data from healthy subjects (Chapter 5) are shown for comparison.

6.6.3 FOXP3+ cells are not proliferating in nonlesional AD skin but show Ki67 expression in lesional skin.

In order to establish the rate of proliferative activity of Tregs in AD skin, we also investigated lesional and nonlesional AD skin sections for coexpression of FOXP3 and the nuclear proliferation marker, Ki67. We found that very low proportions of FOXP3+ cells in nonlesional skin were in cycle (mean 0.91%±0.7, n=7), whereas a significantly higher proportion of FOXP3+ cells from lesional skin were proliferating (mean 5.00%±1.6, n=6, $p=0.0339$).

In comparison, unchallenged (day 0) healthy skin shows virtually no Ki67 expression in any cells in perivascular infiltrates (not shown). Following subcutaneous challenge of healthy skin with PPD, a higher degree of Ki67 expression is seen among FOXP3+ cells (day 3: mean 9.7%±1.1; day 7: mean 8.1%±2.5; day 14: mean 7.9%±1.8; see Chapter 5). Nonlesional skin displayed a significantly lower degree of proliferation than that seen during a secondary immune response (nonlesional AD skin compared to healthy challenged skin: day 3, $p<0.0001$; day 7, $p=0.017$; day 14, $p=0.003$) whereas the degree of proliferation seen among FOXP3+ cells in lesional AD skin is not significantly different from healthy challenged skin (lesional AD skin compared to healthy challenged skin: day 3, $p=0.067$; day 7, $p= 0.3343$; day 14, $p= 0.2639$), although lower (see Figure 6.9C and Chapter 5).

A.



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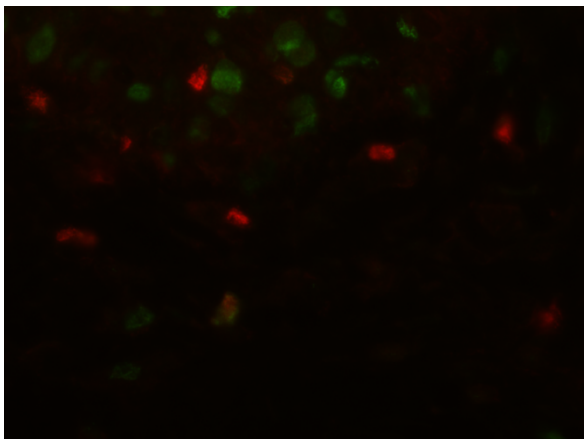

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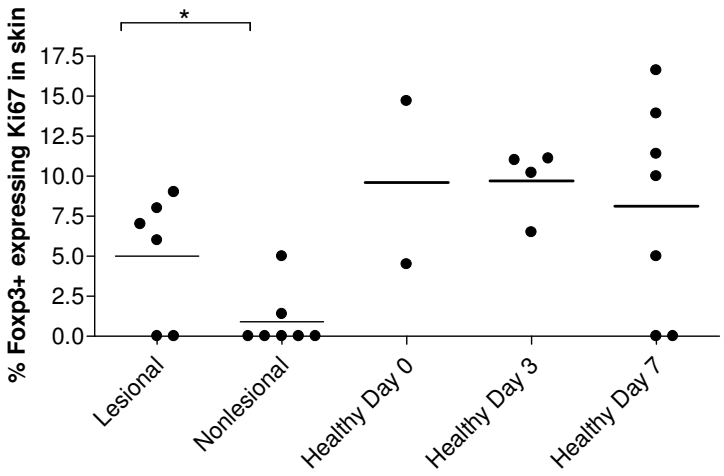

Figure 6.9. FOXP3+ cells in AD patients show a low degree of Ki67 expression in both lesional and nonlesional skin.

A. Representative staining of FOXP3 (red) and Ki67 (green) in nonlesional skin from an AD patient. **B.** Representative staining of FOXP3 (red) and Ki67 (green) from AD lesional skin. **C.** Cumulative data showing the proportion of FOXP3+ cells expressing Ki67 in lesional and nonlesional skin. (n=6 lesional, 7 nonlesional). Data from healthy subjects (Chapter 5) are shown for comparison.

6.7 Discussion

Atopic dermatitis (AD), along with other hypersensitivity conditions such as asthma, is a condition on the increase in the developed world. Worldwide, it affects approximately 20% of children, though on average around 60% of childhood cases resolve by the time the child reaches adulthood [320].

Tregs were implicated in the pathogenesis of AD by the identification of eczema-like symptoms associated with the autoimmune syndrome IPEX (immune dysregulation, polyendocrinopathy, X-linked syndrome), in which the *FOXP3* gene is mutated and the protein non-functional [184, 185]. A high proportion of IPEX sufferers present with eczema-like symptoms [327]; this implies that a deficit of Tregs can make individuals prone to atopic dermatitis, which suggests that Tregs have a role in preventing the disease. A similar condition (scurfy) exists in mice, and the symptoms also often include an eczema-like condition, with scaly, inflamed skin [184, 185]. Despite this circumstantial evidence, a deficit in Treg numbers or function in AD patients has yet to be proven [330-332, 334], though some studies have observed defective suppression by AD Tregs [328]. In this study we aimed to investigate this aspect of AD pathogenesis further, by studying the phenotype, migratory capacity and suppressive ability of AD Tregs and, particularly, the CD45RO+ and CD45RA+ subsets within the Treg pool of AD patients.

We found that a significantly lower proportion of Tregs from AD patients than healthy controls expressed the naïve marker, CD45RA. There is no difference, however, in the proportion of naïve responder T cells, but this cannot rule out the possibility that high levels of inflammation prevalent in these patients may increase the transition of CD45RA+ Tregs to a CD45RO+ phenotype. In addition, however, there was a lower degree of proliferation among these CD45RA+ Tregs, which is surprising: previous studies have indicated that the smaller the naïve subset, the greater the degree of cycling taking place within it [339], so the opposite phenomenon might have been expected. It is possible, however, that CD45RA+ Tregs may convert to CD45RO expression more rapidly in AD patients than in healthy controls, reducing the number of proliferating cells still expressing CD45RA.

We found no difference in the suppressive capacity of AD and healthy Tregs, when used with responders at a 1:1 ratio, either when looking at their effect on proliferation or on cytokine production. However, two areas of interest were unable to be investigated

in this project, due to the difficulty in obtaining significant quantities of blood from AD patients. Firstly, using Tregs at a ratio of 1:1 is not physiological, and defects may be apparent at lower ratios that are missed at this level. Additionally, having identified a potential defect in the number and perhaps proliferation of CD45RA+ Tregs, suppression assays using the two Treg subsets may be highly informative. The suppression assays were also performed using a non-physiological stimulus, anti-CD3, anti-CD28 coated beads. It may be more informative to repeat the experiment using APCs and an antigenic stimulus, perhaps a common allergen such as dust-mite allergen or cat dander, although previous experiments using these stimuli have also found no difference between the capacity of AD and healthy Tregs to suppress responder proliferation [334]. Additional factors *in vivo* that impact on the ability of Tregs to suppress AD responses are also unlikely to be present in the artificial environment of the *in vitro* suppression assay: responder CD4+ T cells may be harder to suppress in AD conditions. Investigation of the function of Tregs from skin would also be useful, as these cells may differ from Tregs isolated from the blood.

Phenotypically, we identified a number of differences between AD and healthy Tregs. CTLA-4 expression is, counterintuitively, more prevalent on the regulatory T cells from atopic individuals. It is possible that this could be the result of activation, since many of the AD patients have widespread disease; it is unlikely to be upregulated to compensate for any defect caused by lack of CD39, as there is no negative correlation between the degrees of expression of the two markers. Although there is no consistent and significant defect in expression of CD39 in AD patients, there does appear to be a subgroup with very low expression of the marker, a phenomenon not present among healthy individuals. This is intriguing, as CD39 has been suggested to play a tolerising role when expressed on Langerhans cells, dendritic cells in the skin [347]. Future investigation of CD39 expression in AD patients, specifically in the skin, would be desirable to establish whether there may in fact be a role for this molecule in the pathogenesis of atopic dermatitis. It was not possible to obtain detailed histories of the AD patients involved in this study, although no connection with age at diagnosis and expression of CD39 or CD45RA could be established, but a longitudinal study may be informative: perhaps those with higher levels of CD39 expression have flare-ups that resolve more rapidly.

Contrary to some previous reports [329], FOXP3+ cells are present and indeed fairly numerous in both lesional and nonlesional AD skin. Although not a significant difference, there tends to be a higher proportion of CD4+ T cells expressing FOXP3 in

nonlesional skin than lesional areas. There is also a significantly higher proportion of CD4+ cells expressing FOXP3 in nonlesional AD skin than in unchallenged normal skin. Nonlesional skin is not conventional, uninflamed skin – almost all of the AD patients recruited had widespread eczema and the biopsied nonlesional areas are highly likely to be regions of resolving or recently-resolved inflammation, which could explain the high levels of FOXP3+ cells shown. AD patients also had large perivascular infiltrates in all sections investigated, which are not observed in unchallenged, normal skin.

Interestingly, although present in high numbers, the FOXP3+ cells in nonlesional skin were not dividing: the proportion expressing Ki67 was consistently significantly lower than that seen throughout a normal secondary cutaneous immune response. Conversely, a higher degree of Ki67 expression was observed in lesional areas, not significantly different from that seen during an immune response. Given the high proportions of FOXP3+ cells seen in nonlesional skin, their low degree of proliferation is a curious observation. Lesional areas are regions of active inflammation, with large infiltrates visible within the skin, and would be expected to show a large degree of proliferation by Treg as well as responder cells. Ki67 was extensively expressed in non-FOXP3+ cells within the AD perivascular infiltrates.

Initial investigations showed a strong degree of proliferation among FOXP3- cells in the AD infiltrates both in lesional and nonlesional skin (Figure 6.9 and not shown). An extension to this investigation would be to stain for Ki67 expression by CD4 cells at lesional and nonlesional sites, to determine whether CD4+ T cells in general in AD infiltrates are dividing, or whether the low rate of proliferation is restricted to Tregs. Future investigations into the proliferative capacity of AD Tregs may be informative; if there is a defect in their ability to divide as much as infiltrating responders, this would be likely to hinder their capacity to control inflammation.

In summary, no functional deficit of AD Tregs could be determined using an APC-free stimulus *in vitro*. However, the unexpectedly low proliferative rate of CD45RA+ Tregs in the blood and FOXP3+ cells in the skin suggests that perhaps there may be some defect in the generation or – given the low cycling rate – the maintenance of thymically-derived Tregs in atopic individuals. If true, this could help to explain why AD is relatively common in children and less so in adults: initially young children have largely naïve cells and are heavily reliant on the naïve subset, but as they grow older and are

exposed to more antigens, Tregs are generated via peripheral conversion, allowing them to ‘grow out’ of the disease.

Chapter 7. Final Discussion and Future Directions

The work presented in this thesis investigated the migration, function and age-related changes of the Treg compartment, particularly in relation to the CD45RO+ and CD45RA+ subsets within it. We investigated the phenotypic characteristics of these cells as well as their *in vivo* migratory behaviour and kinetics during a cutaneous immune response. We then extended our investigations to explore the potential contribution of these cells to the pathogenesis of the hypersensitivity condition, atopic dermatitis.

Early investigations of a suppressive subset of T cells resulted in failure to identify a clear suppressive subpopulation and led to a decline in suppressor cell research [180]. However, in 1995, a suppressive CD25+ subset of CD4+ T cells was identified in mice [181] and the cells termed regulatory T cells. These cells have been extensively investigated since then [182, 183], but it is only recently that the small CD45RA+ subset of Tregs has been investigated specifically in adults [220, 221, 225]. Previous studies have looked at the Treg compartment as a whole [182, 183] or focused on the larger subset of CD45RO+ Tregs [228]. Although it is known that the Treg subset as a whole remains stable [348, 349] or even increases [228, 350] with age in humans, the CD45RA+ subset of Tregs undergoes a sharp decline [221, 351, 352], falling from around 80% of Tregs in cord blood [353] to around 10% by age 20, and thence to around 1% of the Treg pool by the age of 80, as shown in this study. This study aimed in part to investigate similarities and differences between CD45RA+ and CD45RO+ Tregs, to gain a better understanding of the source of memory regulatory T cells and also to establish likely changes in immune regulation with age.

Our initial investigations centred on the composition of the Treg pool throughout life, investigating not only the basic memory and naïve markers CD45RO and CD45RA, but also the recently-postulated recent thymic emigrant (RTE) marker CD31 [69]. We found a sharp decline in the numbers of both CD45RA+ and RTE Tregs during the course of adult life, as has been previously reported [220, 221, 352]. As the numbers of CD45RA+ Tregs declined, the degree of proliferation within this subset increased so that older people had significantly more proliferation among their naïve Tregs than

young adults did; this is likely to be a direct consequence of the smaller CD45RA+ pool in the elderly, as it has been previously reported that smaller pools of naïve cells proliferate to a greater extent [339]. Notably, the degree of proliferation among CD45RO+ Tregs was lower in the old, perhaps because the pool of memory Tregs is by then very highly-differentiated and approaching senescence [353]. This implies that the greater degree of proliferation among CD45RA+ Tregs in the old may have some compensatory effect, to help maintain Treg numbers despite a lower degree of memory cell proliferation.

Overall, the degree of proliferation shown by CD45RO+ was significantly higher than that among CD45RA+ Tregs and remained consistently so within individuals over time. Turnover within the Treg pool as a whole has been shown previously to be rapid *in vivo* [228, 354] but the degree of proliferation among CD45RA+ Tregs was not known at the outset of this investigation. Although much lower than memory Tregs, proliferation among CD45RA+ Tregs is nevertheless significantly higher than that among other naïve CD4+ T cells, suggesting a propensity for frequent cell division among cells that express FOXP3. However, although a high proportion of Treg cells are proliferating at any given time, it is currently impossible to rule out the possibility that there may be a small proportion of long-lived memory Tregs which turn over at a slow rate and are not identified with Ki67 staining. CD45RA+ Tregs did show Ki67 expression to a much lower degree than CD45RO+ Tregs, probably because on stimulation they rapidly convert to express CD45RO. However, within the naive CD45RA+ Treg subset we did note a higher degree of proliferation among CD31+, ‘recent thymic emigrant’ cells, than among CD31- cells. This could be due to residual Ki67 expression from thymic turnover [341]. Additionally, recent studies have shown that some cytokines can allow CD45RA+CD31+ cells to divide without losing expression of CD31 [355], which may partly explain this phenomenon.

CD45RO+ and CD45RA+ Tregs are phenotypically distinct, expressing different levels of the Treg markers CD39 and CTLA-4.This may reflect their different activation states, since both CTLA-4 and CD39 are known to be activation markers as well as Treg-expressed proteins [204, 217]. Although CD45RA+ Tregs express much lower levels of these markers, they appear equally competent at suppressing responder T cell proliferation and cytokine production at a 1:1 ratio. Both molecules have been implicated in Treg function [59, 215, 216, 257] but investigations in this study demonstrated that Tregs not expressing CD39 are able to suppress responses at a timepoint when CD39 has not been significantly upregulated, suggesting that they are

able to function via a CD39-independent mechanism. This is likely to be true for the largely CD39-, CTLA-4- population of CD45RA+ Tregs as well. Preliminary investigations suggest that CD45RA+ Tregs are less able than CD45RO+ cells to suppress responses at lower ratios than 1:1 (data not shown), perhaps as a result of different modes of action able to affect differing numbers of target cells. Further investigation of this area is required to establish to what extent CD45RA+ and CD45RO+ may function differently, as the changing ratios of these cells through adult life could therefore have considerable implications on the maintenance of tolerance into old age. Future studies incorporating siRNA silencing of the CD39 gene in CD45RO+ Tregs may provide further insight into the involvement of this molecule in their function. Since CTLA-4 is not expressed on a large proportion of resting CD45RA+ Tregs, investigations into the kinetics of upregulation of this molecule may also be informative.

An area of some debate at the present time is the proportion of Tregs derived directly from FOXP3+ cells generated in the thymus and the proportion derived from responder CD4+ T cells induced to express FOXP3 in the periphery [356, 357]. This study demonstrates that the potential of CD45RA+ Tregs to become CD45RO+ cells is entirely normal, but cannot provide an answer to the question of relative contributions of the two sources. However, work presented in this thesis proposes a novel mechanism of conversion of responders to acquire suppressive capacity, via anergy induction. Anergised cells showed unresponsiveness to stimulation *in vitro* and were able to suppress the proliferation of control, non-anergised CD4+ T cells. This scenario, in which the induction of anergy results in suppressive cells, lends itself to *in vivo* uses: activated T cells are known to be able to present antigen to each other on upregulated MHC class II molecules and this T:T presentation is also known to lead to anergy [170, 171]. Therefore, in a situation where there is a great deal of inflammation and a large influx of T cells in a confined region, it is highly plausible that these cells could present self- or foreign antigen to one another and thereby induce antigen-specific Tregs. This would be a highly effective safety mechanism preventing immune responses from spreading beyond control. This also has the potential to be exploited in the clinic in the case of allergy or autoimmune diseases: antigen-specific T cells that are the cause or a contributing factor to the disease in question could be removed, anergised and used to bring it specifically to an end without, theoretically, causing generalised immune suppression, as these cells would already be primed to travel to the site of immunopathology.

The distinction between CD45RA+ and CD45RO+ Tregs has been previously investigated to some extent, but their relative migratory behaviour *in vivo* has not yet been studied. However, differing migratory behaviour could indicate different requirements for survival or discrete roles within the immune system. We showed distinct migratory patterns for the two subsets, with CD45RA+ Tregs appearing enriched in the bone marrow and CD45RO expression almost universal among Tregs in the skin. In addition, although CD45RO+ Tregs and responders readily crossed activated dermal endothelium *in vitro*, naïve cells showed no such ability.

CD45RO+ cells can be assumed to have been previously primed, as they have downregulated expression of CD45RA and upregulated CD45RO instead, a post-activation event [65]. A very large proportion of CD45RO+ regulatory T cells is known to express CLA [314, 315]. CD45RA+ Tregs do not express skin-homing markers like CLA and CCR4 to a great extent and in contrast express high levels of the bone marrow-homing chemokine receptor CXCR4, a combination not unexpected in naïve cells [358], and a similar pattern of homing marker expression was seen in CD45RA+ CD4+ responders, although the latter expressed somewhat less of both types of marker. However, naïve Tregs were significantly enriched in the bone marrow, whereas no enrichment was seen among naïve responders. The purpose of such enrichment is not yet known, but it seems reasonable to propose that the bone marrow may play a role in the maturation and development of these undifferentiated cells. It has been shown to have the ability to act as a secondary, as well as a primary, lymphoid organ, providing a site in which antigen presentation can take place [359]; perhaps it plays a role in the initial activation of naïve Tregs. Alternatively, its specific microenvironment [342] may provide an environment conducive to naïve Treg maintenance until they need to be activated and migrate to sites of inflammation. IL-7 has been shown to contribute to the maintenance of naïve conventional T cells [355, 360] but regulatory T cells express low levels of the IL-7 receptor, CD127, and naïve Tregs are no exception. Therefore it is possible that an alternative cytokine may be required to sustain the CD45RA+ Treg pool and that this is prevalent in the bone marrow. Additionally, the cytokine milieu of the bone marrow may allow CD45RA+ Tregs to proliferate without losing expression of CD45RA. Cumulatively, these data indicate that CD45RO+ and CD45RA+ Tregs occupy discrete niches in the immune system.

Identifying Tregs in situations involving a high degree of inflammation is currently a difficult task, due to the phenomenon whereby activated human T cells transiently express FOXP3 [193-196]. Although true Tregs can be identified as distinct from

activated T cells on the basis of demethylation of a promoter region of the FOXP3 gene [218], this is not practical to use as an identifying marker in all situations. This study has confirmed the transient nature of FOXP3 expression in activated T cells, which seems strongly linked to cell division as Ki67 expression by FOXP3+ activated cells was almost universal. However, this thesis demonstrates that FOXP3 expression in the context of a secondary cutaneous response was limited to cells that did not produce cytokines, when restimulated, in any notable quantities, unlike other CD4-expressing cells isolated from the site, which did not express FOXP3 but produced IFN-γ and IL-2 in significant quantities on restimulation. FOXP3+ cells isolated from the site of *in vivo* inflammation therefore appeared to be regulatory T cells, although due to their exceptionally small numbers, their suppressive capacity could not be tested directly. This study therefore suggests that FOXP3 expression *in vivo* in humans is a much more reliable marker of regulatory activity, even at the site of ongoing inflammation, than it is under inflammatory conditions *in vitro*. This has important ramifications for the study of regulatory T cells in humans.

Atopic dermatitis is a hypersensitivity condition that has affected increasing numbers in the developed world in recent decades, particularly children [318, 319]. Atopic conditions have been suggested to be linked to one another – for example, eczema and asthma [322]. If this is so, it means that the study of atopic dermatitis is important not just to gain a better understanding of the causes underlying this disease, but also other conditions such as asthma. Unlike other atopic conditions, however, AD affects a particularly accessible organ: the skin. Work in this thesis indicates that there may indeed be a defect in aspects of Treg function in patients with AD, but in aspects not directly linked to their suppressive function. We found that although Tregs were highly prevalent in the skin of AD patients, particularly in lesional skin but even in skin that was not currently actively inflamed, they were not dividing at a particularly high rate. Additionally, the CD45RA+ regulatory T cell pool was not only much smaller in AD patients than healthy controls but also contained a smaller proportion of dividing cells. Given previous work suggesting that smaller pools of naïve cells show a higher degree of proliferation [339] this is an intriguing finding and, added to the low rate of Treg proliferation in AD lesional skin, a situation that could be equated to acute inflammation and would therefore be expected to entail high levels of proliferation (as shown in Chapter 5), this suggests a potential proliferative defect among AD Tregs. It is of note that, as shown in the photographs in Chapter 6, Ki67 expression among other cells in inflammatory AD infiltrates was high. Nevertheless, no defect in suppression was found in the suppression assays conducted in this study. There may be a number of reasons

for this. First, the suppression assays were performed *in vitro* using an APC-free system, so the influence of antigen presenting cells, or of Tregs on these cells, was not investigated. Additionally, an *in vitro* assay cannot replicate the inflammatory conditions present in the skin of an AD patient during a flare-up of atopic dermatitis. There may be cytokines or other factors present in inflamed AD skin which interfere with Treg suppression. Finally, the AD Tregs used in the study were isolated from the peripheral blood. These cells may behave differently from the Tregs present at the site of inflammation in AD skin.

Additionally, a number of patients displayed a very limited degree of CD39 expression on their Tregs. The scope of this investigation did not extend to CD39 expression on other cell types, but it raises the possibility that there may be a generalised low level of CD39 expression in some people. Although CD39 does not seem to be necessary for Treg-mediated suppression, as shown in work for this thesis, it has been suggested to play a role in cutaneous immune regulation by Langerhans cells [347]. If expression of CD39 by these cells is also shown to be lower, this may be a contributory factor to the cutaneous inflammation seen in AD patients. Also for the future, Th17 cells have been implicated in AD [76] and there is known to be a finely-balanced relationship between Treg and Th17 development [361]. Future investigations could focus on the prevalence of Th17 cells, particularly in the skin at sites of active lesions in AD patients. In addition, investigation of the ability of AD and healthy Tregs to suppress the production of IL-17 by responder T cells may yield interesting results.

This thesis investigated the changes occurring within the Treg pool throughout life and atopic disease. During ageing, the composition of the Treg pool undergoes a fundamental change in that memory CD45RO+ cells become increasingly prevalent. These appear to be largely different from CD45RA+ Tregs, migrating preferentially to different tissues and proliferating to a greater extent, despite having shorter telomeres than their naïve counterparts. We suggest that the two subsets of Tregs, while both important throughout life, may be dominant at different stages. CD45RA+ Tregs are essential during youth to mediate self-specific regulation and to prevent autoimmunity. During ageing, CD45RO+ memory Tregs, particularly those derived from conventional CD4+ T cells, become increasingly important, protecting against chronic inflammation. In atopic dermatitis, CD45RO+ cells form an abnormally high proportion of the peripheral blood Treg pool, yet in many patients, do not express CD39. There also appears to be a defect in proliferation of AD Tregs, despite their strong presence in AD

lesional and nonlesional skin. All these factors may contribute to exacerbate the disease.

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Appendix 1

Appendix 2